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Using N-Acetylcysteine in the Resuscitation of Asphyxiated Newborn Piglets

By

Scott Thomas Johnson

A thesis in partial requirement for the degree of Master of Science

In

Experimental Surgery

DEPARTMENT OF SURGERY

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Dedication

To my parents for their continual encouragement, steadfast support, love and understanding through the years.

Abstract

Current management of asphyxiated neonates consists primarily of supportive care. Much experimental data exists to support the contention that oxygen derived free radicals are responsible for a portion of the morbidity associated with neonatal asphyxia. Despite several studies there are no proven effective therapies to reduce the multi-organ morbidity that results from episodes of severe asphyxia.

We used N-Acetylcysteine, a medication currently used in pediatrics and adult medicine and known to scavenge oxygen free radicals, as an intervention to reduce the morbidity associated with neonatal asphyxia. Our experiment was designed to test the effectiveness of N-Acetylcysteine in four areas: systemic hemodynamics, regional hemodynamics, cellular anti-oxidant capacity, and histologic tissue damage.

Treatment with N-Acetylcysteine 10 min into reoxygenation had a beneficial effect on both systemic and regional hemodynamics. Mean arterial pressure and cardiac index were both improved while mesenteric and renal blood flow were also higher. N-Acetylcysteine increased renal and small bowel tissue levels of glutathione. There were no significant differences in tissue histology between groups. Our results suggest that therapy with N-Acetylcysteine may improve the systemic and regional hemodynamics in asphyxiated neonates.

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List of Symbols

ANOVA- Analysis of Variance

ARDS- Acute Respiratory Distress Syndrome

ARF- Acute Renal Failure

ATP- Adenosine Triphosphate

CFI- Common Carotid Flow Index

CI- Cardiac Index

DIC- Disseminated Intravascular Coagulation

DO_2- Oxygen Delivery

FiO_2- Fractional Inspired Concentration of Oxygen

GI- Gastrointestinal

GSH- Reduced Glutathione

GSSG- Oxidized Glutathione

H_2O_2- Hydrogen Peroxide

HIE- Hypoxic Ischemic Encephalopathy

HR- Heart Rate

IRI- Ischemia Reperfusion Injury

MAP- Mean Arterial Pressure

NAC- N-Acetylcysteine

NEC- Necrotizing Enterocolitis

NO- Nitric Oxide

$O_2^{\bullet-}$ - Superoxide Radical

OFR- Oxygen Free Radicals

$OH^{\bullet}$ - Hydroxyl Radical

$ONOO^-$- Peroxynitrite

PAF- Platelet Activating Factor

PaO_2- Partial Pressure of Arterial Oxygen

PAP- Pulmonary Artery Pressure

RFI- Renal Flow Index

ROS- Reactive Oxygen Species

SOD- Superoxide Dismutase

SMA- Superior Mesenteric Artery

SMAFI- Superior Mesenteric Artery Flow Index

$\dot{V}O_2$- Oxygen Consumption

XDH- Xanthine Dehydrogenase

XO- Xanthine Oxidase

Chapter 1

The Problem of Neonatal Asphyxia

Neonatal asphyxia is a relatively common condition occurring in up to four million infants annually. Up to 25% of these events will prove fatal with another 25% suffering from neurodevelopmental conditions including: cerebral palsy, mental retardation, and epilepsy. (1) The combination of hypoxemia, hypercarbia, and a mixed metabolic and respiratory acidemia contribute to asphyxia. Gilstrap et al (2) suggested that birth asphyxia could be reliably diagnosed with cord blood pH < 7.0 combined with Apgar scores ≤ 3 at five minutes.

Birth asphyxia is thought to be multifactorial in origin. Maternal hypoxemia, maternal hypotension, uterine tetany, premature placental separation, cord compression, drug induced vasospasm and placental insufficiency may all contribute to the development of asphyxia. (1)

Neonatal asphyxia impacts on numerous organ systems (Table 1-1). Despite the many possible systemic effects it remains difficult to discern which neonates will develop severe end organ insult and which organs will be affected.

Severe asphyxia can cause myocardial depression leading to cardiogenic shock. (3) (4) Myocardial injury often includes involvement of the papillary muscles. Clinically this may manifest as right heart failure in the neonate. (5) Post mortem studies of asphyxiated newborns reveals areas of myocardial necrosis. (6) One prospective study in asphyxiated neonates showed that EKG changes correlated with higher serum levels of the isoenzyme CK-MB, evidence suggestive of myocardial injury. Autopsy of study subjects confirmed myocardial necrosis. (7) Asphyxia

induced myocardial dysfunction has been demonstrated in several animal models. (8-10)

Asphyxia has been linked to renal dysfunction in both the neonatal population and experimental models. (14, 15) Luciano et al (16) prospectively studied asphyxiated neonates with acute renal failure and showed they had significantly reduced renal perfusion as measured by doppler. Acute renal failure (ARF) was present in 61% of neonates with severe asphyxia and none with moderate asphyxia in one review. (17) A newborn lamb model of ARF induced by asphyxia led to increased urinary glucose excretion. This was followed by a natriuresis. (14) Natriuresis has also been observed in asphyxiated neonates who develop ARF (16, 18)

The lungs are also impacted by severe asphyxia. Pathologic responses to asphyxia can include pulmonary hypertension, hemorrhage and hyaline membrane disease. Hyaline membrane disease is primarily a disease of premature infants with an incidence inversely proportional to age and weight. (11) Surfactant deficiency is the primary etiology of hyaline membrane disease. Asphyxia may reduce surfactant synthesis rendering neonatal lungs vulnerable. Pulmonary hypertension occurs in 1:500 to 1:700 live births (12) and is perpetuated by asphyxia. Hypoxemia leads to pulmonary vasoconstriction, which in turn results in shunting across the ductus arteriosus. Acidosis results and the cycle of pulmonary hypertension continues. (5) Pulmonary hemorrhage is evident on post-mortem examination in 15% of neonates dying within their first fifteen days of life. (13) Pulmonary hemorrhage is associated with severe asphyxia and has symptoms paralleling hyaline membrane disease.

Necrotizing enterocolitis (NEC) may be related to neonatal asphyxia and will be discussed in chapter 2.

The liver is also affected by asphyxia. Portal blood flow may decrease as the mesenteric vascular resistance increases. (19) Despite a dual blood supply, the liver attempts to compensate by increasing the rate of oxygen extraction. (20) Liver injury may manifest as an increase in liver enzymes, hypoproteinemia, and reduced coagulation factors. (5) Fatal cases of asphyxia have revealed fatty changes, centrilobar necrosis, and hemorrhage. (6)

From a metabolic standpoint neonatal asphyxia is often complicated by hypoglycemia and hypocalcemia. (5) Hypoglycemia has also proven detrimental to neurologic function. (21) However, Yager et al suggested fasting induced hypoglycemia prior to an ischemic insult may confer some neuroprotection. (21) In adults and mature rat models hyperglycemia increases the severity of hypoxia-induced brain damage. (22, 23) Neonatal rat models have produced conflicting results. (24, 25)

Asphyxia effects the hematologic system on several fronts. Platelet number and function are reduced in asphyxia. (26, 27) This may predispose the neonate to hemorrhagic complications. In newborn lambs, asphyxia induced thrombin generation and resulted in consumption of coagulation factors. (27) Thrombocytopenia may be worsened if asphyxia activates the coagulation cascade and disseminated intravascular coagulation ensues. Nako et al (27) measured thrombomodulin levels in asphyxiated neonates compared to controls and demonstrated that endothelial damage may result from asphyxia.

Perhaps the most feared complications of neonatal asphyxia are those to the brain. Of all asphyxia related conditions, those affecting the nervous system are the most likely to persist beyond infancy. Seizures manifest in 60% of asphyxiated newborns. (5) One review of 1200 acidemic term newborns showed an association between neonatal seizures and an umbilical artery pH < 7.0. (29) Seizure activity is also a harbinger of long-term neurologic complications with a 2-5 fold increase in long-term neurologic complications. (5)

Hypoxic ischemic encephalopathy (HIE) is a severe sequela of asphyxia. Between 15 and 20 % of infants with moderate or severe HIE die in the neonatal period and 25-30% of survivors develop severe neurologic conditions such as cerebral palsy and mental retardation. (1) Hypoxic ischemic encephalopathy is divided into mild, moderate and severe categories as described by Sarnat (Table 1-2). (28) Neurologic injury is thought to be mediated by selective neuronal necrosis potentially induced by excess excitatory amino acids. (30) Periventricular leukomalacia is another neurologic lesion attributed to asphyxia in premature neonates. (31) Clinical manifestations may be subtle but over time spastic diplegia develops. Auditory, visual and cognitive dysfunction have also been described. (30)

Neonatal asphyxia is a devastating condition with the potential to affect all organ systems. Numerous animal models have helped to delineate the pathologic response to asphyxia. As our understanding of the pathophysiology grows, so will our ability to treat this morbid and potentially fatal condition.

Table 1-1: Multi-System Involvement in Hypoxic Ischemic Insult

Organ System	Complications
Central Nervous	Seizures, cerebral edema, hemorrhage, syndrome of inappropriate antidiuretic hormone
Cardiovascular	Myocardial injury, cardiogenic shock, dysrhythmias, heart failure
Renal	Renal failure, asphyxiated bladder syndrome,
Gastrointestinal	Gut ischemia, necrotizing enterocolitis hepatic injury: cholestasis, clotting dysfunction, hypoalbuminemia
Pulmonary	Pulmonary hypertension, pulmonary edema or hemorrhage
Hematologic	Thrombocytopenia, disseminated intravascular cogaulation, polycythemia
Metabolic	Hypoglycemia, hypocalcemia, thermoregulation, lactic acidosis

Table 1-2: Sarnat Grading of Hypoxic Ischemic Encephalopathy

Stage	I	II	III
Level of Consciousness	Hyperalert	Lethargy	Stupor or coma
Seizures	No	Common	Uncommon
Stretch Reflexes	Overactive	Overactive	Decreased or absent
Pupils	Mydriasis	Miosis	Variable often unequal
Oculovestibular Reflex	Normal	Overactive	Weak or absent
Duration	< 24 hours	2-14 days	hours-weeks

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Chapter 2

Necrotizing Enterocolitis

Since its initial description by Santulli (1) in 1967 NEC has become an increasingly common diagnosis. With the advent of neonatal intensive care units, survival of critically ill babies has dramatically improved. Necrotizing enterocolitis is one of the most common gastrointestinal emergencies in neonates. The vast majority of cases, up to 90%, occur in babies born prior to 36 weeks gestation. (2) However, NEC also occurs in neonates with asphyxia, cyanotic heart disease and infection. (2) It is estimated to affect between 2000 - 4000 newborns annually with mortality rates ranging between 10-50%. (3) Those who survive an episode of NEC may be left with the morbidity of short bowel syndrome, strictures, malabsorption, cholestasis and fistulas. (4) Most cases of NEC develop within two weeks of birth. The onset of NEC tends to be inversely related to age. (5)

Neonates may present with abdominal distension, bloody stools, pneumatosis or perforation, and shock. In 1978 Bell developed a staging criteria for NEC based on historical, clinical and radiographic data (Table 2-1). (6) Gross examination typically reveals involvement of the terminal ileum and proximal colon. However involvement of the entire gastrointestinal tract (GI) from stomach to anus is possible. Histological lesions are compatible with mucosal edema, hemorrhage, coagulation necrosis, and mucosal ulceration. (3)

Numerous pathogenic theories have been applied to NEC. Santulli et al (7) postulated there were three essential components: mucosal injury, bacteria, and a luminal substrate. Over time theories have been revised in an attempt to explain the pathology.

Initially it was believed that severe respiratory distress, low Apgar score, patent ductus arteriosus, use of umbilical catheters, and early feeding predisposed to the development of NEC. (8) However clinical and epidemiological studies have challenged previous beliefs. (9, 10) Prematurity is the only factor consistently shown to have positive association.

Prematurity of the gastrointestinal tract may play a significant role as immunologic and luminal factors, barrier function and motility are all underdeveloped in the neonate. (3) In 1975 Rieger (11) showed poor antibody response in infants less than 35 weeks gestation when compared to those over 35 weeks. An impaired immune response would leave neonates susceptible to microbes and their resultant toxins. Others have described decreased secretory IgA around Peyers patches in the ileum with increased bacterial translocation in both rabbit and mice models. (12, 13) These findings correlate with the usual localization of NEC to the terminal ileum and proximal colon.

Others have related immature luminal factors to the pathology of NEC. With reduced gastric acidity neonates are readily colonized by enteric pathogens. (3) Some have suggested that reduced activity of the brush border enzymes, notably enterokinase, may suppress the inactivation of bowel altering toxins. (3) This theory is based on pigabel, an adult model of NEC, seen in New Guinea. With a baseline diet replete in antiproteases locals suffer severe enterocolitis after eating raw meat. Bowel damage is mediated by a Clostridium beta toxin that passes thru the gastrointestinal tract without hydrolysis. (1) Interestingly active immunization against the beta toxin prevents the disease process. (14)

An intestinal barrier that is underdeveloped and deficient in mucin has also been implicated in NEC. Reduced mucin content makes the intestine more permeable to large molecules and encourages bacterial adherence to the epithelium. (8) Bacterial translocation may ensue leading to the bacteremia seen with many severe cases of NEC. Uncoordinated peristalsis in neonates may facilitate food intolerance. This may lead to bacterial overgrowth and abdominal bloating. Neu has speculated this may contribute to the development of NEC. (8)

The role of enteral feeding in the pathogenesis of NEC has been ongoing and controversial. Original investigation showed that NEC appeared to occur more frequently in premature babies being enterally fed. This was thought to be secondary to hyperosmolar induced mucosal damage. (4) After the use of hyperosmolar formulas decreased dramatically in neonatal intensive care units, another association was observed: an increase in NEC in those fed more than 20 Kcal/kg/day. This was reinforced by studies showing no increase in NEC when infants were fed small increments (< 10 mL/kg/day). (15, 16) Furthermore in a multicenter trial Lucas et al showed a protective effect of human breast milk when compared to formula. (17) Postulated benefits of breast milk include an abundance of growth factors, enzymes and immunoglobulins, all of which may confer intestinal protection. (4)

The role of bacteria, specifically gram-negative rods, in NEC has yet to be completely elucidated. Numerous studies have suggested that bacteria are essential in the development of NEC. (18, 19) Many neonates with NEC have pneumatosis secondary to bacterial fermentation lending support to an etiologic role. It remains unclear exactly what role bacteria and their resultant toxins play.

Initial anecdotal reports and animal experiments suggested neonatal asphyxia played a pivotal role in the pathogenesis of NEC. (20, 21) This was based on the "diving reflex' theory developed by Scholander (22) and expanded by Lloyd. (23) This theory stated that during episodes of hypoxia blood flow is shunted to the brain and heart at the expense of the viscera. Since then numerous epidemiologic studies have cast doubt on the assumption. (4, 9, 10, 24, 25) Necrotizing enterocolitis does not seem to coincide temporally with hypoxic stress. Furthermore, many cases of NEC occur in infants without evidence of hypoxic stress. Others have noted autoregulatory escape from sustained adrenergic stimulation (basis for diving reflex) in newborn intestine. (26) However, histological evaluation continually reveals finding consistent with an episode of ischemia. (27, 28)

In animal models the transition from fetal to newborn life is accompanied by a dynamic intestinal circulation. Vascular resistance declines precipitously during the first few days, stabilizes, and then increases to a maximal level around postnatal day 30. (29) Vascular tone is mediated by a balance between nitric oxide (NO), myogenic influences and endothelin, with NO playing the primary role in reducing tone. (29) Similar changes are thought to occur in neonates. NO has the effect of increasing blood flow and oxygen delivery, anything interfering with NO production may result in relative intestinal ischemia. Ischemia-reperfusion has been shown to compromise endothelial derived NO production resulting in decreased intestinal flow. (30) Compromised NO production at a cellular level may be important in the development of necrotic intestinal lesions.

Recently attention has turned to inflammatory mediators as potential culprits of bowel necrosis. Fetal enterocytes produce an exaggerated inflammatory response, as measured by interleukin-8 levels, when exposed to inflammatory stimuli. (31) Bowel necrosis has been induced in a rat model using synthetic platelet activating factor (PAF). (32) Other rat models of NEC have shown elevated PAF levels with PAF antagonists reducing the incidence of NEC. (33) Reber (29) postulates that PAF may induce epithelial injury leading to decreased levels of NO and resultant ischemia. In support of this theory MacKendrick (34) found that NO appears to attenuate PAF induced damage as measured by myeloperoxidase activity and histologically. A rat model supplemented with polyunsaturated fatty acids showed a reduced incidence of NEC. In the same experiment PAF receptor expression was reduced as was the measurement of phospholipase A(2)-II mRNA (rate-limiting enzyme for platelet activating factor production). Levels of NO synthase (and presumably NO) were not altered. (35)

Intestinal trefoil factor is secreted by goblet cells to aid in mucosal protection. The mRNA levels of this peptide are lower in newborn rats. (36) A relative deficiency in newborns may render intestinal epithelium susceptible to damage.

Human breast milk has been shown to contain erythropoietin and its receptors have been documented in intestinal tissue. Erythropoietin is thought to protect against intestinal apoptosis. (33) Retrospectively, very low birth weight infants given erythropoietin (for other medical conditions) have a reduced incidence of NEC. (37)

A study out of Columbia suggests a role for probiotics. When compared to historical controls, infants fed daily cultures of Lactobacillus acidophilus and

Bifidobacterium infantis had reduced rates of NEC. (38) A randomized clinical trial recently demonstrated reduced rates of NEC in very low birthweight infants given Lactobacillus and Bifidobacterium. (39) Bifidobacterium, when compared to Escherichia coli or placebo, results in reduced rates of NEC in a rat model. (40)

Despite intense investigation it is still not clear why NEC develops. There is a consistent association with prematurity. The type and rate of neonatal feeding may play a part. Despite lack of epidemiological proof, histological analysis continues to suggest a role for hypoxia. As analysis of various cell mediators proceeds new theories are being put forth that hopefully will elucidate the true etiology of NEC.

Table 2-1: Bell's staging criteria for necrotizing enterocolitis (NEC)

Stage	Classification	Systemic Signs	Intestinal Signs	Radiographic Signs
I	Suspected NEC	Thermal instability apnea, bradycardia, lethargy	Poor feeding, increasing gastric residuals, mild abdominal distension, occult blood in stool	Distension, mild ileus, thickened bowel wall
II	Definite NEC	As above with mild acidosis, thrombocytopenia	As above with marked abdominal distension, absent bowel sounds	As above pneumatosis intestinalis, ascites, bowel edema, portal vein air
III	Advanced NEC	Hypotension, bradycardia, apnea, respiratory and metabolic acidosis, DIC, neutropenia	As above with generalized peritonitis, marked tenderness	As above, pneumoperitoneum

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Chapter 3

Resuscitation With 21% vs. 100% Oxygen

Attempting to correct hypoxia with supplementary oxygen seems intuitive. Since its discovery in the 1770's oxygen has been used in the medical field with beneficial effects. (1) Once 100% oxygen started being used for resuscitation, its role became entrenched in the medical community despite the lack of any evidence. When an association with retinopathy of prematurity was described in the 1950's concerns of oxygen toxicity were raised. (2) Later, in the 1980's the concept of oxidative stress was developed. Since then the role of oxygen toxicity has been increasingly recognized. Data from both experimental animal models and prospective neonatal studies have challenged the concept of resuscitating newborns with 100% oxygen.

The current guidelines of the American Heart Association stipulate that if assisted ventilation is required, 100% oxygen should be used in concert with positive pressure ventilation to achieve a goal of normoxia. If oxygen is not available, room air should be used for resuscitation. (3) Despite any evidence of superiority 100% oxygen continues to be the gold standard for neonatal resuscitation.

One pilot study and two prospective randomized clinical studies have compared room air with 100% oxygen in neonatal resuscitation. (4-6) There were no clinical disadvantages to room air. In fact, the group resuscitated with room air had shorter time to first cry, shorter time to onset of spontaneous respiration, and higher Apgar scores at one minute. Mortality, evidence of hypoxic ischemic encephalopathy, heart rate, acid base status, and oxygen saturation did not differ between the groups. (5) Measures of oxidative stress at 72 hours and 28 days, as assessed by the ratio of reduced to oxidized glutathione, superoxide dismutase and catalase activity were all significantly higher in the group resuscitated with 100% oxygen. (6) This suggests a

protracted, systemic oxidative stress in those given 100% oxygen. Follow up at 18-24 months showed no difference in somatic growth nor evidence of increased neurologic handicap between groups. (7)

Neurologic insult with room air resuscitation has long been a concern. However, numerous animal studies do not show signs of impairment with room air resuscitation. Mongolian gerbils had lower mortality and reduced markers of lipid peroxidation when resuscitated with room air. (8) Furthermore, pathologic examination of gerbil brains revealed that hyperoxia might preferentially damage myelin in the cerebral cortex. (9) Analysis of newborn piglets given room air showed restoration of Na+ / K+ ATPase in the striatum while the 100% group had persistent inhibition. (10) Inhibition of ATPase can lead to leeching of electrolytes and resultant cytotoxic edema. (11) Bagenholm et al (12) showed there was no difference in cerebral hemispheric weight, a surrogate for cerebral damage, in rats resuscitated with 21% and 100% oxygen.

Rootwelt et al (13) looked at a neonatal pig model of resuscitation with 21% and 100% oxygen. Histologic brain morphology did not differ between groups. Another study by the same author examined cerebral blood flow and evoked potentials during resuscitation with 21% vs. 100% oxygen. (14) Cerebral blood flow and forebrain oxygen extraction did not differ between groups resuscitated with 21% and 100% oxygen. Somatosensory evoked potentials did also not differ between groups. This led the authors to conclude that 21% is not inferior to 100% oxygen when applied to the reoxygenation of hypoxic newborn pigs. Furthermore another group using pneumothoraces to induce hypoxia, found impaired early neurologic

outcome in the 100% oxygen group. There were no differences in markers of oxidative stress or in cerebral histopathology. (15) Further data supports the assertion that 21% is not inferior to 100% oxygen during neonatal resuscitation. Dogs undergoing normothermic cardiac arrest followed by resuscitation with either 21% or 100% oxygen fared better neurologically in the first 24 hours when 21% oxygen waws given. (16)

Hypoxanthine, a breakdown product of adenosine triphosphate (ATP) and marker of hypoxia, has been measured in neonatal pig models. There were no differences in peak plasma and cerebrospinal fluid levels post hypoxia, nor any difference in the plasma elimination rates, in groups resuscitated with 21% and 100% oxygen. (13) Another model measuring hypoxanthine concentration in the cerebral cortex revealed higher concentrations in animals resuscitated with 100% oxygen. (17) Hypoxanthine levels in plasma and femoral muscle did not differ. The authors hypothesize that hyperoxic resuscitation may damage the blood brain barrier or alter energy metabolism in the cerebral cortex.

Another neonatal pig model assessed hydrogen peroxide production in neutrophils. It was thought that production of reactive oxygen metabolites might be proportional to partial arterial pressure of oxygen. Results confirmed the hypothesis. Both arterial and cerebral venous hydrogen peroxide concentration increased during hypoxia. However during reoxygenation hydrogen peroxide levels in the cerebral venous circulation remained elevated in the 100% group, while they were low in the 21% group. During reoxygenation cerebral blood flow was restored equally in both groups. Interestingly, 100% oxygen induced a decrease in oxygen uptake by the

forebrain which was significant at 60 min. (18) This finding had been previously documented in another neonatal pig model. (14)

A model of hypoxemia and meconium aspiration syndrome and resuscitation with either room air or pure oxygen showed no difference between groups. Mean arterial pressure, pulmonary arterial pressure, cardiac index, base excess and plasma hypoxanthine levels were all assessed. (19) This model extends the spectrum of room air resuscitation to include neonates suffering from meconium aspiration. Another model from the same institution looked at the effect on the pulmonary vasculature of using 21% vs. 100% oxygen during resuscitation. Hypoxic vasoconstriction returned to levels near baseline in 21% and 100% resuscitated groups. Plasma endothelin –1 (a vasoactive peptide) levels normalized in both groups. From this study it appears that 21% oxygen is equally effective in reversing hypoxic mediated pulmonary vasoconstriction. (20)

Other studies have documented a direct relationship between free radical generation and hyperoxic exposure. A recent neonatal pig model demonstrated an increase in lung chemiluminescence and thus an increase in reactive oxygen species in pigs resuscitated with 100% oxygen when compared to the room air resuscitated group. (21) Other studies have also suggested that the generation of oxygen derived free radicals is proportional to the oxygen content during resuscitation. (19)

Regional blood flow following hypoxemia and resuscitation with either 21% or 100% was characterized by Rootwelt et al. (22) During hypoxia blood was preferentially shunted to the brain and heart in both groups. After 1 hr resuscitation there was no significant difference in blood flow, nor oxygen delivery to the heart,

liver, kidney or intestine when comparing the 21% and 100% groups. (22) Using a neonatal piglet model of hypoxia-reoxygenation, Haase et al demonstrated no histologic difference in the ileum between groups resuscitated with either 21%, 50% or 100% oxygen. In fact, the only 2 cases of gross pneumatosis were seen in the group resuscitated with 100% oxygen. (23)

Numerous neonatal pig models have demonstrated no difference in base deficit, pH, mean arterial pressure and cardiac index between groups resuscitated with 21% and 100% oxygen. (13, 17, 19, 20) Some experimental data demonstrates a transient improvement in cardiac index in animals resuscitated with 21% oxygen. (24) Twenty-one percent oxygen appears at minimum, equally efficacious when considering basic hemodynamic and systemic parameters.

Several animal models have been used to assess the effects of resuscitation with room air in comparison to 100% oxygen. The results seem to be consistent: there is no evidence that resuscitating with 100% oxygen is superior to room air. In contrast, certain indices suggest that room air may confer some benefits. More studies are needed to determine the precise role, if any, of supplemental oxygen in neonatal resuscitation.

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Chapter 4

Oxygen Free Radicals in Neonatal Asphyxia

Cells require oxygen to survive. Individual tissues tolerate interruptions in oxygen delivery differently. However, the end result is the same: cellular dysfunction leading to cellular edema and ultimately cell death.

Reperfusing previously hypoxic tissue has some paradoxical effects. Increased blood flow increases oxygen delivery and helps to wash out toxic metabolites, but it may also induce further injury. (1) Parks and Granger (2), using a cat model of intestinal ischemia, showed that 3 hours of ischemia followed by 1 hour of reperfusion resulted in more severe injury than four hours of ischemia alone. Along the same lines others have shown a reduction in gastric and skeletal muscle injury when ischemic tissues are reperfused with hypoxic blood. (3, 4)

Oxygen toxicity, in part, has been attributed to the development of oxygen free radicals (OFR). Initial papers in the late 1960's described an accumulation of OFR in cardiac ischemia. (5) It was hypothesized that tissue injury occurred during the re-establishment of tissue oxygenation following a period of hypoxia. (6) This became known as the 'oxygen paradox'. (5) With underdeveloped intracellular oxidative defence systems preterm neonates may be at increased risk of oxidative damage. (7) Oxygen free radicals have been implicated in a multitude of neonatal diseases such as: chronic lung disease (bronchopulmonary dysplasia), retinopathy of prematurity, persistent patency of the ductus arteriosus, pulmonary hypertension, NEC and intraventricular hemorrhage. (6, 8) An understanding of the generation of OFR is necessary to appreciate their role in disease.

Oxygen free radicals consist of species derived from molecular oxygen with an unpaired electron. The unstable single electron has a high affinity for stable

biological molecules such as: lipids, proteins and nucleic acids. Reactions with biological molecules yield several unstable products that can lead to cellular damage and destruction. Peroxidation of cell membranes with subsequent loss of cellular integrity and function is a major mechanism of OFR induced dysfunction. (9) Damage is evident in mitochondria isolated from organs subjected to ischemia-reperfusion as shown by increased markers of oxidant stress. (10) Mitochondrial dysfunction is thought to be secondary to lipid peroxidation. (11)

Ischemia-reperfusion injury (IRI) is believed to generate a plethora of OFR. As ischemia progresses and creates an oxygen deficiency cells are forced to switch to anaerobic metabolism. Anaerobic glycolysis is much less efficient than aerobic oxidative phosphorylation, producing only 6% as much adenosine triphosphate (ATP) per molecule of glucose. Consequently ATP stores are rapidly depleted. High-energy phosphate bonds in ATP are cleaved generating hypoxanthine (Figure 4-1). During anaerobic metabolism glucose is metabolized to pyruvate and lactic acid. The accumulation of lactic acid contributes to a metabolic acidosis commonly seen in asphyxiated newborns. As ATP stores are exhausted, energy dependent transmembrane ion pumps fail, cells lose their ability to maintain ion gradients and cytotoxic edema ensues. Loss of the cellular calcium gradient raises intracellular calcium levels. High calcium levels activate numerous cellular pathways, some of which generate OFR. (12) Morris et al (13) documented a burst of oxygen radicals during the immediate reperfusion period.

The xanthine oxidase (XO) system was implicated early as a contributor to the generation of OFR. In the cell xanthine oxidoreductase can exist in two enzymatic

variants, xanthine dehydrogenase (XDH) and xanthine oxidase (XO). It is responsible for the conversion of hypoxanthine to xanthine and xanthine to uric acid. During normal cellular metabolism XDH predominates using hypoxanthine, water and NAD^+ to produce xanthine. (14) However, during ischemia XDH is converted to its oxidant producing variant XO. Xanthine oxidase uses molecular oxygen to generate the reactive oxygen species hydrogen peroxide (H_2O_2) and superoxide anion ($O_2^{\bullet}$-) during the breakdown of hypoxanthine (Figure 4-2). (5) Experimentally, levels of hypoxanthine increase following episodes of hypoxia. (15) Thus ischemia creates an ideal environment for the generation of OFR: increased amounts of substrate (hypoxanthine) and new enzyme activity (xanthine oxidoreductase).

The XO system is most active in the fetal liver and intestine. (16) Xanthine oxidase levels are just above the threshold of detection in human myocardium and brain. (5) Increased plasma hypoxanthine levels were found in a group of asphyxiated newborns when compared to normal controls. (17) This finding led the authors to suggest that hypoxanthine levels could be a marker for the degree of hypoxia. Furthermore, in ischemia-reperfusion experiments, measured levels of circulating XO are significantly elevated. (18) Supnet et al (19) showed elevated umbilical cord blood levels of XO in sick newborns. Higher XO levels were related to worse outcomes.

The $O_2^{\bullet}$- generated by the XO system is itself cytotoxic. However, $O_2^{\bullet}$- is also necessary for the generation of the more potent hydroxyl radical ($OH^{\bullet}$). Hydroxyl radicals can be formed via a variety of reactions. In the Haber-Weiss reaction $O_2^{\bullet}$- and H_2O_2 combine to form $OH^{\bullet}$. In the presence of transition metals (such as iron),

H_2O_2 will accept an electron to generate $OH^{\bullet}$. The metal is then reoxidized by $O_2^{\bullet}$-. This is known as the Fenton reaction (Figure 4-3). (9)

Nitric oxide is an endogenous ubiquitous molecule produced by endothelial cells, macrophages and neutrophils. (20) It is generated by the enzyme NO synthase. During normal physiologic states NO is present in low levels and acts to regulate blood flow, platelet aggregation, neural activity and cellular endothelial adhesion. Nitric oxide is also a vital part of the immune response system. (21)

While NO is vital to maintain normal physiology, under certain circumstances it can exert toxic effects. Its toxicity is thought to result from the reaction between $O_2^{\bullet}$- and NO. This is a diffusion-limited reaction: each molecular collision results in a reaction and irreversible generation of an intermediate peroxynitrite anion ($ONOO^-$). Nitrites and nitrates serve as end products (Figure 4-4). (9) During the pathologic condition of ischemia and reperfusion the synthesis of both NO and $O_2^{\bullet}$- are greatly increased. This facilitates the generation of $ONOO^-$. (22)

Peroxynitrite is a highly reactive compound produced in shock and ischemia reperfusion models. (23, 24) It is very unstable and capable of reacting with a variety of biologic materials including: proteins, DNA, membrane phospholipids, deoxyribose and methionine. (25) Peroxynitrite is capable of crossing cellular membranes in its protonated form, peroxynitrous acid, or through anion channels. (26) Peroxynitrite has been shown to oxidize tissue sulfhydral groups significantly faster (in the order of 1000-2000 times) than H_2O_2. Oxidation of sulfhydrals is a known toxicity of OFR as this interferes with enzyme active sites and / or alters the native conformation of proteins. (20) Radi et al (25) showed that $ONOO^-$ was capable

of inhibiting mitochondrial respiration by inactivating ATPase and components of the electron transport chain. Peroxynitrite also led to an increased production of H_2O_2 by the mitochondria. Mitochondrial damage may be one of the mechanisms by which $ONOO^-$ exerts its cytotoxic effects.

Peroxynitrite also functions to nitrosylate tyrosine groups of proteins, a pathological modification which has been documented in many disease processes. Generation of antibodies to $ONOO^-$ first documented its presence in atherosclerotic vessels. (26) Since then $ONOO^-$ nitrosylated metabolites have been found in the lungs of infants with respiratory disease, in intestinal tissue of neonates with NEC, and in the serum of infants with chronic lung disease. (27) Using spectroscopic determination of dityrosine, Yasmin et al (28) documented the production of $ONOO^-$ in isolated rat hearts undergoing ischemia reperfusion.

Superoxide radicals are also generated by neutrophils via a membrane surface NADPH oxidase system. Normally quiescent, the system is activated by either cytokines or bacteria to generate a 'respiratory burst' reducing oxygen to $O_2^{\bullet}$- and H_2O_2. (9) Myeloperoxidase (an enzyme generated by neutrophils) is capable of generating hypochlorous acid, a highly reactive compound, from H_2O_2 and chloride ions (Figure 4-5). Hypochlorous acid has a greater cellular toxicity than either $O_2^{\bullet}$- or H_2O_2. (29)

Post ischemia neutrophils, as part of the inflammatory response, aggregate in reperfused tissue. Neutrophils adhere to the endothelium creating a microenvironment of highly toxic agents. (30) Neutrophils are recruited to areas of ischemia reperfusion by a variety of chemoattractants. Levels of leukotrine B_4 and platelet activating factor

have both been correlated with neutrophil infiltration. (14) Considerable evidence exists to support the role of neutrophils in microvascular injury post ischemia reperfusion. Reperfusion induced vascular injury is reduced in neutropenic animals as well as those treated with antibodies to CD18 which prevent endothelial leukocyte adhesion. (14) Myocardial infarct size and acute cardiac failure are both reduced in leukocyte-depleted models. (9)

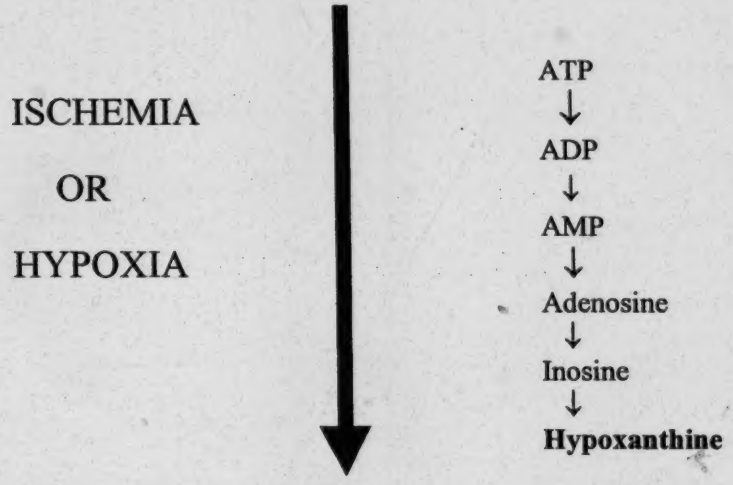
Under normal physiologic conditions the mitochondrial electron transport chain reduces oxygen to water, generating ATP in the process. During the process a small percentage of the oxygen is converted to the $O_2^{\bullet}$-. Most of the $O_2^{\bullet}$- generated is scavenged by mitochondrial antioxidant systems. However, in some pathological conditions, for example ischemia-reperfusion, mitochondrial defences are overwhelmed. The net result is an accumulation of OFR such as $O_2^{\bullet}$- and subsequently a generation of H_2O_2 and $OH^{\bullet}$. (10)

In one study mitochondria in rat brains subjected to ischemia generated increased amounts of $OH^{\bullet}$. (11) Mitochondrial inhibitors decreased the production of $OH^{\bullet}$. Ambrosio et al (31) looked at rabbit hearts subjected to ischemia-reperfusion. He found that mitochondria generated significant amounts of OFR. As in the above experiment OFR formation was reduced by the introduction of a mitochondrial respiration inhibitor.

Oxygen free radicals have been well studied over the past twenty years. Our understanding of OFR generation has increased rapidly. New experiments have been designed to elucidate the role of certain OFR in specific disease processes. While

there have been some promising results, no final common pathway has been proposed for any of the neonatal conditions thought to be caused by OFR.

Figure 4-1: Generation of Hypoxanthine



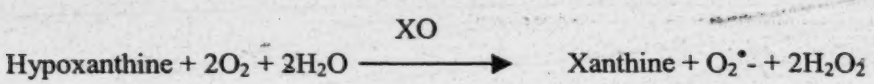
As ischemia progresses high energy phosphate bonds are cleaved. In the process hypoxanthine is generated as an end product.

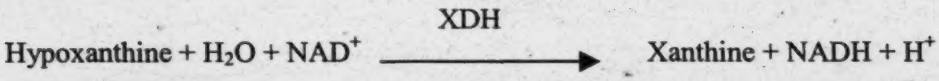
ATP- Adenosine Triphosphate

ADP- Adenosine Diphosphate

AMP- Adenosine Monophosphate

Figure 4-2: Xanthine Oxidase System





The breakdown of hypoxanthine by xanthine oxidase and xanthine dehydrogenase.

Xanthine oxidase is responsible for generating reactive oxygen species.

XO- Xanthine oxidase

XDH- Xanthine dehydrogenase

Figure 4-3: Generation of Hydroxyl Radicals

I. Haber-Weiss Reaction

$$O_2^{\bullet}- + H_2O_2 \longrightarrow O_2 + OH^{\bullet} + OH^-$$

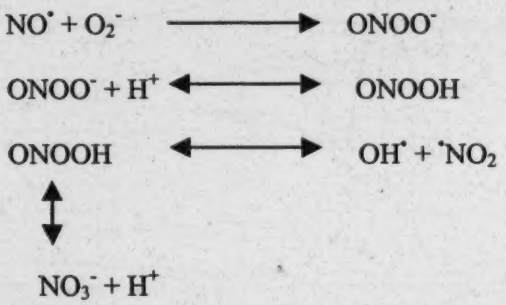
II. Fenton Reaction

$$O_2^{\bullet}- + Fe^{3+} \longrightarrow O_2 + Fe^{2+}$$

$$H_2O_2 + Fe^{2+} \longrightarrow OH^{\bullet} + OH^- + Fe^{3+}$$

Hydroxyl radicals can be generated via two independent reactions. The Haber-Weiss reaction occurs independently of transition metals.

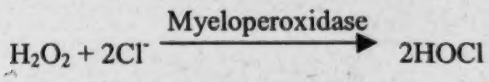
Figure 4-4: Generation on Hydroxyl Radicals and Peroxynitrite



Sequence responsible for the generation of the highly unstable peroxynitrite anion.

Hydroxyl radicals are also generated during the cascade.

Figure 4-5: Generation of Hypochlorous Acid by Neutrophils



Activated neutrophils use myeloperoxidase to generate hypochlorous acid which is extremely toxic to cells.

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Chapter 5

N-Acetylcysteine as an Antioxidant

Cellular oxidants exact damage in many ways. Free radicals are capable of destroying enzymes by inducing conformational changes, peroxidizing cell membranes breaking down cell integrity, oxidizing DNA and interacting with cell signal transduction. (1, 2) To counteract these effects there are a variety of cellular antioxidant defense systems in place. Glutathione is one of the main intracellular antioxidants. (3) In addition, vitamins A, C, and E all have antioxidant properties. (4) Several enzymes, notably superoxide dismutase, catalase and glutathione peroxidase, are a vital part of cellular antioxidant systems. Superoxide dismutase (SOD) provides some protection against the $O_2^{\bullet}$- by catalyzing the conversion from $O_2^{\bullet}$- to H_2O_2. Catalase can then convert H_2O_2 to molecular oxygen and water, preventing the formation of toxic OH•. Glutathione peroxidase catalyzes the oxidation of reduced glutathione (GSH) by hydrogen peroxide to form oxidized glutathione (GSSG) and water. Glutathione reductase then converts GSSG back to GSH. (4) (5) Catalyse, glutathione peroxidase and glutathione reductase are all found in the cytosol of most cells (Figure 5-1). (5)

There are other components to cellular antioxidant defense systems. Selenium is a co-factor for the activity of glutathione peroxidase, and thus plays an important role in the natural defense system. (4) Free radical scavengers provide a second line of defense. These hydrogen atom donors serve to make free radicals less toxic. Some described scavengers include: uric acid, bilirubin, mannitol, methionine, carotenoids and mucin. (4, 5)

Interventions targeting cellular defense mechanisms have been successful in reducing ischemia-reperfusion injury (IRI). Allopurinol, an analogue of

hypoxanthine, inhibits XO via a sequence of reactions. Allopurinol attenuated IRI in newborn lamb hearts by reducing afterload and thus increasing cardiac output when compared to controls. (6) There were no measurable changes in cardiac contractility. In cat intestine pretreated with allopurinol, Parks and Granger (7) showed a reduction in IRI, as measured by intestinal permeability. Pretreatment with allopurinol prior to intestinal IRI reduced the influx of neutrophils and attenuated the drop in levels of reduced glutathione. (5) In another study geared towards inhibiting the XO pathway, cats were fed a tungsten-supplemented diet (tungsten inactivates XO by binding to an active site). As expected mucosal XO activity was significantly reduced, but so was the measured IRI. (8)

The two other endogenously occurring antioxidant systems, SOD and catalase are more difficult to study experimentally. As the molecules are large they do not readily pass through cell membranes. Consequently they must be either conjugated or encapsulated to gain cellular entry. In a canine model SOD and catalase improved myocardial function post IRI. (9) Other investigators have duplicated this finding. (10) Infusion of SOD and catalase reduced infarct size in a rat model of cerebral ischemia. (11) In another experiment, pretreating intestinal samples with SOD reduced IRI as measured by the presence of villous and crypt necrosis. (12) In a cat model of graded intestinal hypoperfusion, Schoenberg et al (13) showed a protective effect of SOD on the intestinal mucosa.

Other interventions introducing free radical scavengers have also been tried. Dimethyl sulfoxide and dimethylthiourea are both $OH^{\cdot}$ scavengers with the benefit of being membrane permeable. (14) In one study of canine myocardial IRI,

dimethylthiourea but not dimethyl sulfoxide reduced myocardial infarcts. (15) Further experiments are needed to clarify any role for these compounds.

Iron chelating agents have been used to decrease the amount of iron available as a catalyst during the Haber-Weiss reaction. (Figure 4-3) In neonatal rabbit hearts, deferoxamine (an iron chelator) improved contractile function post IRI. Damage as measured by creatine phosphokinase was also reduced, as was the generation of free radicals. (16) In a rat model of intestinal ischemia and reperfusion, pretreatment with deferoxamine reduced the severity of intestinal injury. (17)

One study looked at the role of vitamins C and E in preventing antioxidant stress. By inducing hypoxia in a fetal rabbit they showed that vitamins C and E had a protective effect on fetal myocardial damage. Total fetal antioxidant potential was also increased in the treatment group. (18) Similarly, isolated newborn piglet hearts pretreated with vitamin E and subjected to ischemic arrest followed by reperfusion had improved post-ischemic recovery when compared to controls. (19) Human volunteers given 8 weeks of vitamin E supplementation had altered monocyte production. In response to endotoxin their monocytes had reduced generation of OFR and interleukin-1B. However, (20) under severe oxidant stress vitamin C can reverse roles and function as a pro-oxidant. (21)

Glutathione and glutathione peroxidase are vital components of the cellular antioxidant defense. Manipulations of this system have been tried in many experiments. In a rat heart model of ischemia-reperfusion, infusion of glutathione resulted in a concentration dependent improvement in mechanical function. (22) Furthermore, concentrations of dityrosine (a marker of peroxynitrite) were reduced in

the GSH treated group. Mice that over-express glutathione peroxidase show resistance to myocardial ischemia-reperfusion by improved contractile recovery and reduced infarct size. (23) Conversely, mice that do not express glutathione peroxidase are more susceptible to cardiac IRI. (24) One other model of IRI in rat hearts showed a protective role for GSH infused during the hypoxic period. (25)

N-Acetylcysteine is a complex thiol molecule. The use of NAC is predicated on its antioxidant activity. In vivo NAC is converted to L-cysteine, which is then used to replace intracellular stores of glutathione (Figure 5-2). The sulfhydryl group in NAC also provides some direct antioxidant activity. (21)

N-Acetylcysteine has been utilized in many experimental and clinical situations. It was originally used as a mucolytic agent in patients with cystic fibrosis, serving to break sulfide links between large molecules and DNA in viscous secretions. (26) Around the same time NAC was discovered to be beneficial in acetaminophen overdose. (27) NAC counteracted the liver toxicity by replenishing glutathione stores and preventing generation of hepatotoxic metabolites.

N-Acetylcysteine has also reduced the oxidative stress injury associated with reperfusion during liver transplant by decreasing the levels of circulating cellular adhesion molecules. (28) Using a rat model of hepatic ischemia-reperfusion, Nakano et al (29) used NAC infusions prior to ischemia to demonstrate a protective effect on hepatocytes. The NAC infusion group had decreased levels of hepatocellular enzymes (markers of hepatic injury) and increased bile production in comparison to controls. The NAC group also demonstrated higher concentrations of GSH and GSSG post-reperfusion.

The use of NAC in the prevention of renal failure has also been studied. In a cohort of male rats infusion of NAC one hour prior to and one hour following renal ischemia resulted in significant protection. Glomerular filtration rate was substantially improved at both one and seven days. (30) The renal protective effect of NAC has been clinically studied in the prevention of contrast-induced nephropathy. In a group of patients with chronic renal insufficiency, Tepel et al (31) first showed that hydration and prophylactically administered NAC could reduce the deterioration in renal function associated with intravenous contrast. This finding was replicated in a group with moderate renal insufficiency undergoing coronary angiography. (32) It is believed that NAC exerts its protective effect by inhibiting oxidative damage and improving renal hemodynamics by increasing the concentration of NO. (32)

Forman et al, using a rat model of coronary ischemia showed that NAC reduced myocardial stunning. (33) Infarct size and generation of free radicals however were not affected. In cultured cardiac myocytes NAC decreased the generation of ROS. (34) Using a murine endothelial cell line subjected to hypoxia-reoxygenation, Isowa et al (35) demonstrated a protective effect for NAC in terms of cellular viability.

Several experimental and clinical models assessing the impact of NAC on the respiratory system have been developed. In a rat model of acute lung injury intra-tracheal administration of NAC in a liposomal suspension resulted in reduced lung damage immediately and at prolonged periods. (36) In another rat model, acute lung injury induced by intra-tracheal administration of interleukin-1 was attenuated by an intravenous infusion of NAC. Benefits were seen both if the NAC was given before

or 2.5 hours after induction of the lung injury. (37) Wagner et al (38) used a canine model to investigate the effect of NAC on oxygen toxicity. Dogs were ventilated with 100% oxygen for 54 hours. Those given NAC had reduced pulmonary pressures, better lung compliance, less alveolar and interstitial edema and reduced pulmonary white blood cell infiltration. In an animal model of acute respiratory distress syndrome rats given NAC fared significantly better. There was a reduction in the amount of fibrin in precapillary vessels as well as reduced alveolar edema. (39) In a clinical trial investigating the impact of NAC on the development of acute respiratory distress syndrome, the NAC treatment group had improved oxygenation and a decreased need for ventilatory support. The development of acute respiratory distress syndrome and mortality were not affected. (40) Another clinical trial of patients with diagnosed acute respiratory distress syndrome showed an increase in cardiac index and repletion of red blood cell glutathione stores. The duration of acute lung injury was also shortened. Mortality however, was not affected. (41) In a recent neonatal clinical trial an infusion of NAC during the first week of life failed to reduce the incidence of bronchopulmonary dysplasia or death. (42)

The systemic effects of NAC have also been investigated. In one prospective clinical trial NAC was administered to patients undergoing major abdominal surgery. Inflammatory mediators were then measured for three days. N-Acetylcysteine was found to reduce the levels of C-reactive protein, but not procalcitonin nor microalbuminuria. (43) The clinical significance of reduced levels of C-reactive protein is unknown. One trial of septic patients investigated the ability of NAC to decrease cellular adhesion molecules and inflammatory mediators at the

transcriptional level. Inhibition of transcriptional activators was evident in the NAC treated group. Levels of the inflammatory mediator interleukin-8 were also reduced. Neither interleukin-6 nor intercellular adhesion molecules were affected. (44) Another study looking at patients in early septic shock analyzed the effect of NAC on the generation of oxidative stress. Compared to the control group, patients given NAC had lower indices of peroxidation. (45)

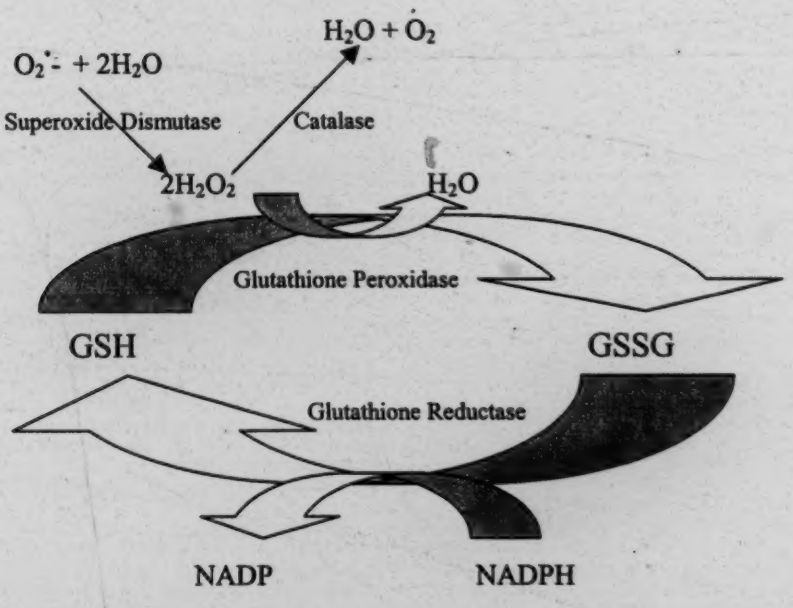
Using NAC in the setting of intestinal IRI has generated some positive results. One rat model showed intestinal necrosis could be reduced by up to 60% in the NAC treated (428 mg/kg/hr) group. (46) Another rat model pretreated with a NAC bolus (20 mg/kg) followed by an infusion (20 mg/kg/hr) showed wide-ranging effects. N-Acetylcysteine reduced the histological ileal injury. Tissue staining for oxidant-induced injury was decreased in the NAC treated group. Similarly, myeloperoxidase levels were also reduced in the treatment group. (47) Olanders et al (48) used a rat model of intestinal IRI to assess local and systemic effects of NAC treatment (150 mg/kg). Although the treatment group did not show any change in cellular adhesion molecules, reductions in bowel and lung endothelial permeability, lung neutrophil accumulation and plasma interleukin-1B levels were all observed.

The direct antioxidant activity of NAC has been studied. In one experiment NAC was found to interact with and neutralize hypochlorous acid (derived from neutrophils), and $OH^{\bullet}$. Interaction with H_2O_2 was observed, but very slowly. N-Acetylcysteine was found to be a poor scavenger of $O_2^{\bullet}$-. (49)

N-Acetylcysteine has a wide range of biologic effects, many of which are still being elucidated. N-Acetylcysteine is attractive as an intervention, not only because

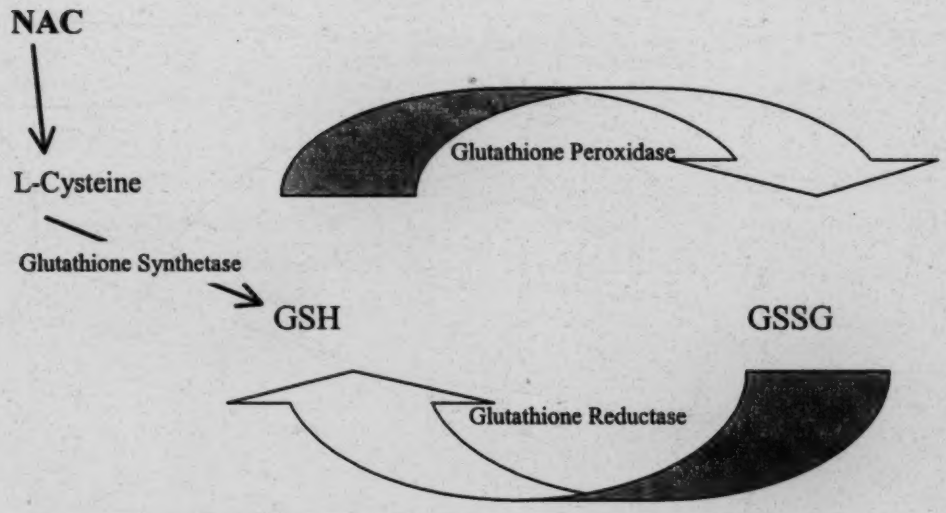
of its diverse actions, but also due to its ease of administration and safety profile. It can be administered orally, intravenously, or in nebulized form. Doses range from less than 1 gram for nebulized solutions to 15 grams for adult acetaminophen overdoses. (50) Years of animal and clinical experimentation have verified its safety profile. The most common side effects in clinical trials are nausea and emesis during intravenous boluses. Skin rashes have also been described. (51) N-Acetylcysteine may alter the vasorelaxation properties of vessels by increasing the availability of NO, resulting in hypotension. (47) Angioedema and anaphylactiod reactions have also been described in a few cases. (52) Serious side effects are rare and NAC remains a valuable clinical medication. Delineation of further clinical applications requires further experimentation to define the precise role of NAC.

Figure 5-1: Cellular Antioxidant Systems



The three main intracellular antioxidant systems are depicted above. Reaction with glutathione neutralizes oxygen free radicals. Glutathione is then regenerated within the cell.

Figure 5-2: Exogenous Administration of N-Acetylcysteine



Exogenously administered N-acetylcysteine is converted to glutathione and may act to increase depleted cellular stores of reduced glutathione. This has the effect of increasing cellular antioxidant capacity.

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Chapter 6

Surgical Swine Models, Models of Necrotizing Enterocolitis and Hemodynamic Measurements

I. Swine in an Acute Neonatal Surgical Model

The history of using pigs as surgical models dates back as far as 1543. (1) The use of pigs in experimental surgery has numerous advantages over other species. Swine are readily available, but more importantly they have many anatomic and physiologic similarities to humans. Swine maturity at birth parallels human infants making it an ideal neonatal model. (1)

Human and swine neonates share many anatomic, physiologic and developmental similarities in their cardiovascular, pulmonary, intestinal and renal systems. As such swine have served as models of: heart, pancreatic, kidney, small bowel and liver transplantation, wound healing, endocrine studies of reproduction, experimental gastrointestinal procedures, and interventional techniques in radiology. (1) Swine have also been used to replace dogs in operative classes.

As a cardiovascular model swine share many similarities with humans. Coronary anatomy follows an end artery distribution that results in territorial ischemia. (2) Consequently swine as models of myocardial ischemia are well established. (3) Developmental regulation of the cardiovascular system in terms of central nervous system input is similar in human neonates and neonatal piglets. (4) Hemodynamic parameters and responses to stressors such as: hemorrhage, hypoxia, and hypercapnia in piglets resemble those of human newborns. (5, 6)

Newborn piglet pulmonary vasculature is similar to that of a human newborn, as is the remodeling it undergoes in the postnatal period. (7) Vigorous vasoconstriction in response to hypoxia makes the piglet an ideal model for studying pulmonary hypertension and neonatal hypoxia. (7) Models of neonatal persistent

pulmonary hypertension of the newborn have been successfully developed in piglets. (8) In newborn humans the ductus arteriosus typically closes at birth in response to elevated concentrations of inspired oxygen. In newborn piglets however, the ductus arteriosus remains open anatomically. Despite anatomical patency, several studies have documented functional closure with lack of blood flow through the ductus arteriosus in newborn pigs. (9, 10)

From a renal standpoint, other than dwarf water buffalo, pigs are the only animal with an extra renal pelvis and multipyramidal kidneys like humans. Renal development and physiology also parallel man. This makes pigs an ideal model for studying the kidney and its related pathophysiology. (11) With an anatomic structure similar to humans, neonatal pigs have been used to study autonomic regulation of renal blood flow. Renal response to different medications including: indomethacin, furosemide, and hydrochlorothiazide have also been evaluated to assess age related differences in response to various drugs. (11)

Piglet response to intra-abdominal sepsis and frank shock has been studied to determine how resuscitative efforts are best directed. (12, 13) Hypotension, as induced by sequential cardiac tamponade, results in selective splanchnic vasoconstriction. Clinically this is a model of non-occlusive mesenteric ischemia. (4) Interestingly, in contrast to other animal models, swine do not appear to become bacteremic in the setting of severe hypotension. (4) This prevents septic shock from confounding hypoxia induced cardiogenic hypotension.

Neonatal swine intestinal physiology is similar to humans in several respects. (1) Models mimicking NEC by inducing fecal peritonitis have been developed. (13).

Gross and histological evidence of NEC has been induced in neonatal pigs using the combination of hypoxia and hypothermia. (14) The effects of total parental nutrition on the hepatic, pulmonary and splenic system have been evaluated in the neonatal pig and applied to human neonates. (15)

There are however, some differences between neonatal human intestine and neonatal swine intestine. Piglet intestines undergo much more rapid growth during the first few days of life and for the first day piglets are capable absorbing intact proteins through the intestine. (16)

Human and piglet livers share many microscopic and ultrastructural similarities. (17, 18) Neonatal piglets models have been used to study the impact of acute and chronic total parenteral nutrition on the liver. (19, 20) Changes similar to those seen in human neonates were produced.

Numerous studies have documented baseline swine hematological values, and many are similar between human neonates and young swine. (21) Due to the extensive use of swine in biomedical research, baseline hematologic and chemistry values for swine of varying degrees of maturity have been tabulated. (22)

For an acute surgical model, swine can be anesthetized with inhalational agents to facilitate surgical manipulation. Inhalants are favorable as they are titrateable, a benefit during surgical procedures. Halothane results in a decrease in cardiac output and blood pressure. This effect is dose dependent and is seen with other modern agents such as isoflurane. (23, 24). While halothane can be toxic to the liver, isoflurane appears to avoid hepatic metabolism and the resultant hepatotoxicity. (23) Halothane can also sensitize the myocardium to catecholamine-

induced arrhythmias. Adding nitrous oxide to the inhalant mixture and using the lowest possible levels of halothane for adequate anesthesia can attenuate all these effects. (24) Muscle relaxants, benzodiazepines, ketamine, thiopental, and narcotics, all of which have clinical applications, have also been proven safe in pigs. (23, 25) Knowledge of pharmacologic effects in newborn swine helps to approximate the clinical scenario of a heavily sedated newborn in the neonatal intensive care unit.

As described above the pig is an ideal model for surgical experimentation based on similar anatomic, developmental and physiologic systems.

II. Models of Necrotizing Enterocolitis

Numerous surgical models of NEC have been utilized. The models can be divided into four main categories: (i) hypoxia / ischemia reperfusion (ii) luminal nutrients (iii) inflammatory mediators (iv) combinations of the above.

An early study of asphyxiated neonatal piglets showed histologic changes similar to those described in the early NEC reports. (26) Necrotizing enterocolitis was induced in low-birth weight piglets by ligation of mesenteric vascular arcades for 48 hours. The spectrum of injury ranged from mucosal erosion to perforation. Damage was more significant when more vessels were ligated and when vessels supplying the ileo-colic region were ligated. Similar manipulation of older pigs did not result in cellular damage. (27) Changes consistent with NEC have been documented in term neonatal piglets; pneumatosis intestinalis and histologic small intestinal injury similar to that seen in NEC has been documented in a model of hypoxia-reoxygenation. (28)

A different model of global ischemia-reperfusion demonstrated ischemic bowel lesions consistent with those seen in NEC. (29)

In comparison to piglets, young rats are less resistant to bowel ischemia. One model of superior mesenteric artery (SMA) occlusion for 1 minute produced bowel necrosis in nearly 50% of the group at one week. (30)

An adult rabbit model of NEC was developed by Clark et al (31) using acidified bovine casein. Mucosal hemorrhage was documented using ^{51}Cr labeled red blood cells. Further investigation of the model revealed loss of the mucosal villi (early necrosis) and increased intestinal permeability. (32) Only 16 hours after treatment with the acidified casein nearly 40% of the rabbits had hemorrhagic necrosis. (33)

Recently models of bowel necrosis have been developed using inflammatory mediators in adult rats and mice. At small doses PAF induces focal small bowel necrosis within hours. At high doses the entire small bowel may infarct. (34) As rat platelets are refractory to PAF the necrosis is likely secondary to a vasoactive effect, not a thromboembolic phenomenon. High doses of lipopolysaccharide also cause bowel necrosis in rats. Lipopolysaccharide will act synergistically with PAF, inducing bowel necrosis at smaller doses. Another mediator, tumor necrosis factor will cause bowel injury and the addition of lipopolysaccharide seems to have a synergistic effect exacerbating bowel necrosis. (35) In a similar rat model a combination of low dose lipopolysaccharide and hypoxia resulted in significant bowel necrosis. (34)

Barlow et al (36) developed a NEC model in newborn rats using a combination of hypoxia, formula feeding, and *Klebsiella* inoculation. Necrotizing

enterocolitis developed independent of the bacterial inoculation. The model was expanded one year later showing that repeated episodes of cold stress are equally as effective as hypoxia in inducing NEC in formula fed *Klebsiella* inoculated neonatal rats. (37) Formula was added to a hypoxic model of neonatal dogs to induce NEC. The dogs underwent hypoxia followed by randomization to either colostrum or formula feedings. The formula fed group had pathologic changes under light microscopy similar to NEC at 24 hours. (38) A similar model of NEC using asphyxia and formula feeding has been employed in neonatal rats. (39)

III. Measurement of Blood Flow

There are several methods to experimentally measure blood flow. For the purpose of acute surgical experimentation perivascular flow probes provide accurate documentation of second to second variations in blood flow. Ultrasonic transit-time probes measure volume flow in a vessel by passing signals through the flowing blood. The transducers then convert the ultrasonic wave to an electrical signal and metered output. (40) Ultrasonic flow probes have been used extensively in research and validated by numerous studies. (41, 42), (43)

Electromagnetic flow probes are also used to quantify blood flow. The probe works on the principle that an electrical field is induced when a conductor such as blood passes through a magnetic field. The size of the electrical field created is proportional to both the magnetic field (which is generated by the probe) and the velocity of the conductor. The voltage of the electrical field is measured by the probe

and converted to a flow output. (44) Electromagnetic probes do require a tighter fit around the vessel and can be hampered by electrical interference.

The microsphere technique can also be used to determine organ blood flow. It can be especially helpful to measure flow to organs perfused by multiple arteries. This particular technique is based on the principle that the small radiolabelled microspheres follow the same distribution as red blood cells and that the spheres remain in the organ being studied. Flow is determined by calculating the percent of tracer present in the organ over the total injected. This technique gives a static picture of flow. (44)

One other method used to measure blood flow is the laser doppler flow meter. Optical fibers in the flow probes emit a pulse of laser light. Part of the light is reflected back to a sensor which records the amount of time elapsed between the emitted and returned signals. Using the Doppler principle this information is then used to calculate flow measurements. Laser Doppler flow probes can be used for continuous monitoring and or recording.

For our experiment we selected ultrasonic transit-time flow probes based on their ability to accurately measure acute variations in regional blood flow. Transit-time flow probes are not constrictive around vessels, conductance is via a gel medium. This eliminates the potential for flow probe induced vasospasm which can impact flow readings.

IV. Experimental Hypotheses

Based on the information put forth we investigated the effects of N-acetylcysteine in a neonatal model of asphyxia-reoxygenation. Our experiments were designed to test the hemodynamic and biochemical effects along with the impact on tissue histology of N-acetylcysteine in our model of asphyxia-reoxygenation. Our central hypothesis was that by decreasing oxygen free radical generation we would be able to reduce the end organ injury associated with hypoxia-reoxygenation. We therefore put forth the following specific hypotheses:

1. Treatment with NAC will reduce the cardiac morbidity associated with our model of hypoxia-reoxygenation.
2. Treatment with NAC will reduce the oxidative stress and improve renal perfusion without adverse systemic hemodynamic effects.
3. Treatment with NAC will attenuate intestinal hypoxia-reoxygenation injury.
4. Treatment with NAC will improve carotid artery blood flow during hypoxia-reoxygenation.

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Chapter 7

Systemic Effects of N-Acetylcysteine in a Neonatal Piglet Model of Hypoxia-Reoxygenation

Introduction

The majority of clinically asphyxiated newborns undergoing successful resuscitation will suffer from end organ injury. (1) While the heart is not the most frequent site of injury, cardiac damage does occur in a significant number of asphyxiated newborns. A prospective study of asphyxiated newborns revealed cardiac involvement in 29% of the neonates as manifested by EKG changes, murmurs or overt heart failure. (2) Other neonates will have cardiovascular collapse and require inotropes secondary to myocardial dysfunction. (1) In a post-mortem analysis of neonates who succumbed to asphyxia 62% demonstrated significant cardiac pathology. (3)

Investigation into the cardiac morbidity associated with neonatal asphyxia has been extensive. Many studies have focused on 'myocardial stunning' and the generation of oxygen free radicals (OFR) during reperfusion of previously ischemic tissue. (4-7) Oxygen free radicals are highly unstable species that have the potential to interact with cells destabilizing their function. (8) Interventions have been used with limited success to decrease the production of OFR in an attempt to reduce the cardiac morbidity associated with ischemia-reperfusion injury. (9-11) It has been difficult to maintain drug potency while delivering adequate concentration to the appropriate tissues. To date no single intervention has generated results that translate into a reproducible, reliable clinical therapy.

Cellular antioxidant defence comes in a variety of forms. Glutathione is one of the main intra-cellular antioxidant systems. (12) In isolated human and rat hearts ischemia-reperfusion resulted in a reduction in cardiac glutathione levels. (13) Another model used glutathione infusion to reduce the myocardial injury associated with

ischemia-reperfusion. (14) N-Acetylcysteine (NAC) acts as a direct antioxidant via its terminal thiol group and also as a precursor to glutathione. (15) N-Acetylcysteine has an excellent safety profile and is used in a variety of clinical conditions. It is standard of care for acetaminophen overdose and has recently gained recognition in the prevention of contrast induced nephropathy. (16, 17) Treatment with NAC has previously been shown to increase cellular stores of glutathione. (18)

Based on the current understanding of ischemia-reperfusion injury and NAC's known properties, we designed an experiment to test the hypothesis that treatment with NAC will reduce the cardiac injuries in a neonatal piglet model of hypoxia-reoxygenation.

Materials and Methods

Animals

Large white piglets (1-4 day old, 1.4-2.2 kg), were obtained from the university farm on the day of the experiment. All experiments adhered to the regulation of the Canadian Council of Animal Care and were approved by the Health Science Animal Welfare Committee at the University of Alberta. The minimum acceptable arterial pre-operative hemoglobin was 7 g/ dL.

Anesthesia and Preparation of Animals

Piglets were initially anesthetized with 5% isoflurane which was then titrated between 2-3% to ensure adequate anesthesia. A pulse oximeter (Nellcor, Hayward, CA) was used to monitor oxygen saturation. Temperature was monitored rectally and maintained between 38.5°C and 40°C with an overhead warmer and heating blankets.

After an airway was surgically established the pig was mechanically ventilated with pressure-controlled assisted ventilation (Sechrist infant ventilator model IV-100, Sechrist Industries Inc. Anaheim, CA) using pressures of 20/4 cm H_2O and a rate of 18 breaths per minute. The fractionated inspired oxygen concentration (FiO_2) was set between 21-25% to maintain oxygen saturation between 88-95%. Oxygen concentration was measured by an Ohmeda 5100 oxygen monitor (Ohmeda Medical, Laurel, MD). Inhalational anesthesia was discontinued after the animal was mechanically ventilated. Anesthesia was maintained with boluses of fentanyl (20 μg) and pancuronium (0.6 mg) followed by IV infusions of pancuronium (0.05-0.1mg/kg/hr), fentanyl (5-15μg/kg/hr), and midazolam (0.1-0.2mg/kg/hr). Further boluses were given as necessary to maintain anaesthesia. Maintenance fluids throughout the experiment were 20 mL/hr of 10% dextrose and 4 mL/hr of normal saline. Bicarbonate was not given at any point during the experiment. Sampled blood in excess of what was required was returned to the animal. Total blood sampling during the experiment amounted to approximately 8cc.

Surgical Procedure

The femoral artery and vein were exposed via a right groin incision. A double lumen catheter was placed in the femoral vein (3.5F or 5F Argyle™, Sherwood Medical Co, St Louis, MO) and advanced 15 cm to the level of the right atrium for infusion of intravenous (IV) fluid. A single lumen catheter was placed in the femoral artery, 3.5F or 5F Argyle™, and advanced 5 cm into the infra-renal aorta. Advancement of catheters to 15 and 5 cm was based on previous experiments. Both lines were connected to pressure transducers for measurement of central venous pressure (CVP) and mean systemic

arterial pressure (MAP) respectively. A tracheotomy was performed and a 3.5mm internal diameter endotracheal tube (Portex Inc, Wilmington, MA) was inserted into the trachea. Mechanical ventilation was started. A left anterior thoracotomy at the 4th interspace was performed. The pericardium was incised exposing the main pulmonary artery. A 20 G Insyte-W™ (Becton Dickinson Infusion Therapy Systems Inc., Sandy, UT) was placed into the pulmonary artery, secured and attached to a pressure transducer for measurement of mean pulmonary artery pressure (PAP). A 6 mm Transonic® flow probe (6SB) was then placed around the pulmonary artery.

Hemodynamic Calculations

Cardiac output was corrected for individual pig weight and expressed in mL/kg/min as cardiac index (CI). Arterial oxygen content was estimated by the following: (1.36 X Hemoglobin X oxygen saturation) + (0.002 X PaO_2). Systemic oxygen delivery (DO_2) was approximated by the product of CI and arterial oxygen content. Systemic oxygen consumption ($\dot{V}O_2$) was calculated in the following manner: CI X [1.36 X hemoglobin X (arterial oxygen saturation – systemic venous oxygen saturation)].

Stabilization

After the surgical procedure all piglets were stabilized for 30-45 min. Inspired oxygen concentration (21-25%) and respiratory rate (12-22) were adjusted until the following criteria were met: i.) PaO_2 60-100 mmHg ii.) $PaCO_2$ 35-45 mmHg iii.) pH >

7.35. Stability was defined as hemodynamic variation < 10% for 15 minutes while fulfilling the above blood gas criteria.

Monitoring

Oxygen saturation, heart rate (HR), MAP, CVP, CI and PAP were continuously monitored and recorded throughout the experiment. Pressure and flow readings were digitized using a DT 2801-A converter board (Data Translation, ON) in a Dell 425E personal computer. Asyst custom software was used to continuously record all signals on a hard disk. Recordings at specific intervals were taken for two minutes and averaged.

Experimental Protocol

Piglets were block randomized into one of four groups: surgical sham, hypoxic control, early treatment with NAC and late treatment with NAC.

The surgical sham group was ventilated at 21% oxygen for 6 hr. The 3 remaining groups were subjected to 2 hr of hypoxia by progressively decreasing the FiO_2 by increasing the amount of inhaled nitrogen. FiO_2 was manipulated to keep PaO_2 between 20 and 40 mmHg while reducing the cardiac output to 40% of baseline. Piglets were then resuscitated with 100% O_2 for 1 hr followed by 21-25% O_2 for 3 hr. The IV NAC was prepared by diluting NAC (200 mg/mL) 1:1 in 10% dextrose. Treatment or placebo of equal volume 10% dextrose was then given. The early treatment group was given an IV bolus (150 mg/kg) of NAC at the time of reoxygenation. The late treatment group was given an IV bolus (150 mg/kg) of NAC 10 min into reoxygenation. Both

treatment groups received an IV infusion of NAC (100 mg/kg/hr) 30 min from the time of reoxygenation which was continued for the duration of the experiment. The infusion was prepared by diluting 5 mL of NAC (200 mg/mL) into 95 mL 10% dextrose. The hypoxic control received an equal volume placebo of 10% dextrose. At the conclusion of the experiment the piglet was euthanized with 240 mg IV pentobarbitol. Post-mortem examination confirmed placement of all catheters, flow probes and patency of vessels. Tissue samples from the left ventricle were removed, snap frozen and placed in liquid nitrogen. Additional samples were placed in 10% formalin for histological analysis.

Physiologic Measurements

All data was recorded in a blinded fashion. Treatment was given in unmarked bags and the experimenter was not aware of group assignments. Baseline recordings of HR, MAP, CVP, PAP, pulmonary blood flows, arterial blood gases (ABL 500, Radiometer Medical, Denmark), arterial and venous co-oximetry (OSM3 Hemoximeter, Radiometer Medical, Denmark) were taken. Hemodynamic, flow probe, arterial blood gas, and venous co-oximetry measurements were recorded at 30 min intervals during hypoxia and at t=10, 60 and 240 mins of reoxygenation. During reoxygenation hypotension (MAP < 35 mmHg) was treated with a fluid bolus of 10 mL/kg. This was repeated as necessary.

Histology

Tissue samples were collected at the termination of the experiment and placed in 10% formalin. Tissue from the left ventricle was embedded in paraffin blocks,

sectioned and stained with hematoxylin and eosin. Two pathologists (Lawrence Jewell, Graham Slack), who were blinded to the randomization, independently analyzed slides.

Biochemical Analysis

Myocardial levels of oxidized and reduced glutathione of the left ventricle were determined using a glutathione assay kit (catalog number 703002, Cayman Chemical, Ann Arbor, MI). Briefly, 50 mg ±10% were homogenized in 500 µL of cold buffer consisting of 0.4 M 2-(N-morpholino) ethanesulphonic acid, 0.1 M phosphate, and 2 mM EDTA, pH 6.0 (MES). The sample was then centrifuged at 10,000 x g for 15 minutes at 4°C. The supernatant was removed and stored at -20°C. Deproteination of all samples was accomplished by adding an equal volume of 10 % metaphosphoric acid (MPA) to the sample. The solution was mixed and allowed to stand for 5 minutes, then centrifuged at >2,000 g for 5 minutes. The 4 M triethanolamine (TEAM) reagent was added to the samples (50 ul/ml of supernatant). The assay was performed by adding the assay cocktail [MES buffer, cofactor mixture, enzyme mixture, and 5,5'-dithiobis-2-nitrobenzoic acid (DTNB)] to the sample. The microplate was incubated in the dark on an orbital shaker and the absorbance was measured at 25 minutes at 405 nm using a microplate reader (Titertek Multiscan PULS, MKII, Labsystems, Finland). The total glutathione concentration was calculated using a standard curve. Oxidized glutathione was measured by mixing deproteinated samples with 1 M 2-vinylpyridine and incubating for one hour at room temperature. The assay then was performed as described above. Levels of reduced glutathione (GSH) were determined by subtracting oxidized glutathione (GSSG) from the total glutathione levels. GSH and GSSG content were expressed as umol/L.

Data Analysis

Data was analyzed using SigmaStat2.0 for windows (Jandel Corporation, San Rafael CA). All results are expressed as mean ± standard error of mean unless otherwise stated. Using variances from previous experiments with the same model, (19) sample size of 6 in each group was determined with a power calculation (β=0.2) to determine a 50% difference in CI. Experimental groups were compared at different time points using one-way analysis of variance (ANOVA). Repeated measure ANOVA was used to analyze differences over time. For non-parametric data, analysis was done on ranks. Post hoc analysis was completed using the Tukey test or Dunn's method for pairwise comparison of parametric and non-parametric data, respectively. Correlation was analyzed using the Pearson Product Moment test. Histologic injury was compared using the Fisher Exact test. For all analysis significance was defined as $p < 0.05$.

Results

Baseline hemodynamic and arterial blood gas characteristics did not differ between groups (Table 7-1). Heart rate did not differ between groups throughout the experiment at the predefined time points.

Two hours of hypoxia resulted in significant reductions in MAP (61±7 vs. 32±4, 26±4, 24±2 mmHg, for sham vs. hypoxic control, early and late treatment, respectively, $p<0.05$) (Figure 7-1). Blood pressure recovered immediately during reoxygenation with no differences between groups. After 1 hr reoxygenation the hypoxic control and early treatment group had a significantly lower MAP than the surgical sham (35±2 and 36±3

vs. 57±7 mmHg, respectively, $p<0.05$) while the late treatment group had a higher blood pressure (vs. end of hypoxia, $p<0.05$) and was not significantly different from the sham (42±2 vs. 57+7 mmHg, $p>0.05$). Blood pressure of the late treatment group then fell and there were no significant differences between the 3 treatment groups or the surgical sham after 4 hr reoxygenation. Fluid boluses post hypoxia were required in all treatment groups to maintain MAP. After 4 hr reoxygenation the differences between groups were not significant, but the late treatment group had required less fluid than both the hypoxic control and early treatment groups (8.3±0.3 vs. 25±0.8 and 20±0.5 mL/kg, for late vs. control and early treatment respectively, $p=0.13$, $\beta=0.77$).

Cardiac index was also significantly depressed after 2 hr of hypoxia (163±13 vs. 71±8, 93±7, 88±8 mL/kg/min, for sham vs. control, early and late treatment respectively, $p<0.05$) (Figure 7-2). Cardiac index rebounded immediately within 10 min reoxygenation. There was no difference between groups at either 10 min or 1 hr reoxygenation. After 4 hr reoxygenation CI in the late treatment group was closer to the sham than both the hypoxic control and early treatment group (165±21vs. 141±13, 101±17 and 98±23 mL/kg/min, for sham vs. late, control and early treatment respectively, $p=0.06$, $\beta=0.59$). In the late treatment group, compared to the end of hypoxia there was a significant preservation of CI during the later phases of reoxygenation. This was not observed in the hypoxic control or early treatment group where the CI at the end of the experiment was not significantly different from the values at the end of hypoxia (Figure 7-2).

Two hours of hypoxia generated a severe metabolic acidosis. Arterial pH remained significantly depressed reaching a nadir at 10 min reoxygenation (7.34±0.02

vs.6.92±0.05, 6.90±0.03, 6.91±0.08, for sham vs. control, early and late treatment respectively, $p<0.05$). Recovery occurred during further reoxygenation. However, after 1 hr reoxygenation (with $PaCO_2$ ranging between 39 and 41 mmHg), pH in both the hypoxic control and early treatment group but not that of the late treatment group remained significantly depressed compared to the surgical sham (7.10±0.04, 7.11±0.03 and 7.18±0.06 vs. 7.35±0.01, respectively, $p<0.05$). After 4 hr reoxygenation the pH of the hypoxic control and late treatment groups had recovered but that of the early treatment group remained significantly depressed in relation to the surgical sham (7.17±0.02 vs. 7.33±0.02, respectively, $p<0.05$).

Pulmonary artery pressure increased significantly during the first hr of hypoxia (43±4, 43±2, 45±5 vs. 29±1 mmHg, for control, early and late treatment vs. sham respectively, $p<0.05$) then decreased during the second hour of hypoxia and was not significantly different from the surgical sham at 2 hr of hypoxia (Figure 7-3). Resuscitation with 100% oxygen decreased PAP, which returned to near baseline levels after 1 hr reoxygenation, then increased slightly for the duration of the experiment. There were no differences between groups during reoxygenation.

Systemic DO_2 was similar among groups at baseline (Table 7-2). It then declined in the groups exposed to hypoxia. Ten minutes of reoxygenation restored systemic DO_2 to near baseline levels. Oxygen delivery then declined through the duration of the experiment. After 4 hr reoxygenation there was no difference in systemic DO_2 between treatment groups whereas systemic DO_2 of the early treatment group was lower than the surgical sham ($p<0.05$). Systemic $\dot{V}O_2$ was similar among groups at baseline. Hypoxia resulted in a reduction in systemic $\dot{V}O_2$ in all three

treatment groups. Systemic $\dot{V}O_2$ remained significantly lower than the sham at 10 min reoxygenation and gradually improved over the course of reoxygenation. However, in the early treatment group after 4 hr reoxygenation systemic $\dot{V}O_2$ remained lower compared to the sham.

After 4 hr reoxygenation left ventricle myocardial levels of total glutathione were significantly elevated in the early treatment group when compared to the hypoxic control (172±9 vs. 135±7 umol/L respectively, $p<0.05$) (Figure 7-4). Total glutathione levels in the late treatment group were also elevated although not statistically significant. Tissue levels of GSSG did not differ between groups, nor did the redox ratio (GSH: GSSG) (data not shown). After 4 hr reoxygenation there was no significant correlation between CI, systemic DO_2 and myocardial levels of total glutathione or GSSG.

Under light microscopy there were no histological changes in the left ventricle.

Discussion

The systemic morbidity associated with neonatal asphyxia is significant. Neurological, cardiovascular, pulmonary, renal and intestinal manifestations have all been described. (1) In this experiment we examined an intervention to reduce the cardiac morbidity associated with our model of neonatal asphyxia. The neonatal piglet model of asphyxia has been well established and published in various forms. (20-22) The newborn piglet is an acceptable model; developmental regulation of the cardiovascular system by the central nervous system is similar in human neonates. (23)

Piglet hemodynamic parameters and responses to stressors such as: hemorrhage, hypoxia, and hypercapnia are similar to human newborns. (24, 25)

Two hours of hypoxia resulted in a progressive reduction in MAP, CI, pH and systemic DO_2. Hypoxia induced depression of the cardiovascular system was not surprising. These findings have been mirrored in other experiments, as have the improvements in systemic hemodynamics following reoxygenation. (21, 22) One study in particular demonstrated a reduction in mechanical myocardial function as measured OFR production increased. (26) Other experiments exposing the myocardium to high concentrations of OFR have also resulted in reduced cardiac function. (4, 27, 28) Some models have shown improvements in cardiac function with interventions designed to reduce OFR generation. (4, 29-31)

N-Acetylcysteine has been used in clinical practice for several decades and is safe to administer to both children and adults. (32) There is little published data on the safety of NAC in the neonatal population. Our study provides some evidence that NAC could be used in asphyxiated neonates without adverse hemodynamic effects. On the contrary, treatment with NAC 10 min into reoxygenation seemed to improve MAP, CI and systemic DO_2 without having any impact on HR or PAP. Late treatment with NAC also resulted in less fluid boluses to maintain systemic blood pressure. We speculate improvement in hemodynamics following treatment with NAC could be due to its direct role as an OFR scavenger. (33) Experiments by Bolli (4), Yasmin (7) and Garlick (6) have all demonstrated the generation of OFR following myocardial reperfusion. Timing the NAC intervention with reoxygenation and the resultant OFR development may have been beneficial for the cardiovascular system. Giving NAC at the onset of

reoxygenation may not be as beneficial as OFR production has not yet peaked. Studies quantifying OFR production show a peak between 2 and 4 minutes into reoxygenation and continued for upto 3 hr. (6, 34) Unfortunately the current experiment was not designed to directly assay OFR. One limitation of our study is we did not analyze tissue or serum samples for direct markers of oxidative stress.

Our intervention did increase left ventricle tissue levels of GSH. However, levels were highest in the early treatment group while the greatest clinical effect was documented in the late treatment group. The treatment effect may not have been directly related to intracellular GSH content. Rat hearts subjected to ischemia-reperfusion had improved mechanical function if infused with GSH. This benefit was independent of intracellular GSH content. (14) N-Acetylcysteine, a precursor to intracellular GSH, is also known to directly detoxify OFR as it contains a terminal sulfhydryl group. (15)

Our goal of intervening to reduce end organ damage was not realized in this experiment. None of the histologic specimens demonstrated any changes. Our protocol of 4 hr reoxygenation likely did not allow sufficient time for pathological changes to occur under light microscopy. Given the significant hemodynamic depression one would expect ultrastructural changes in myocardial tissue. However, we did not analyze our samples using electron microscopy. Other investigators have shown ischemic changes in the myocardium using protocols allowing longer intervals of recovery post-asphyxia. Using a model of umbilical occlusion, myocardial necrosis was observed in 2 of 7 severely asphyxiated fetal lambs 72 hr after the asphyxia insult. (35) Clinicopathological series of asphyxiated newborns have also confirmed cardiac involvement. (3, 36)

Treatment with NAC 10 min after resuscitation is without any major adverse hemodynamic effects in our model of severe asphyxia-reoxygenation and resulted in improved clinical parameters. Further study is required to fully define the role of NAC in protecting the myocardium after neonatal asphyxia.

Table 7-1: Baseline hemodynamic and physiologic characteristics* of piglets

	Surgical Sham	Hypoxic Control	Early Treatment	Late Treatment
HR (bpm)	203±16	220±11	239±7	217±12
MAP (mmHg)	70±5	63±5	68±1	71±4
PAP (mmHg)	25±1.4	29±4.3	26±0.6	26±2.3
CVP (mmHg)	3±1	4±1	5±1	4±1
CI (mL/kg/min)	229±27	195±20	233±17	226±14
pH	7.39±0.02	7.39±0.01	7.39±0.008	7.40±0.01
$PaCO_2$ (mmHg)	39±1	40±1	41±1	40±2
PaO_2 (mmHg)	68±2	79±10	67±2	64±2

*No differences between groups

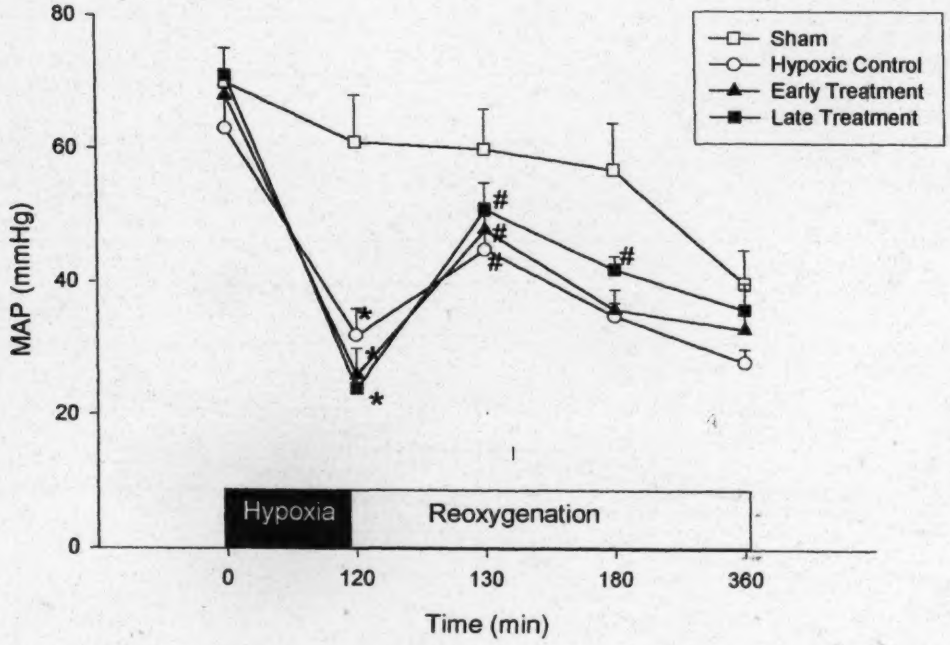
HR- heart rate, bpm- beats per minute, MAP- mean arterial pressure, PAP- pulmonary artery pressure, CVP- central venous pressure, CI- cardiac index

Table 7-2: Systemic oxygen metabolism during hypoxia-reoxygenation

	Baseline	End Hypoxia	10 min Reoxygenation	4 hr Reoxygenation
Systemic Oxygen Delivery (mLO_2/kg /min)				
Sham	26±3.2	18±1.6	18±1.7	18±2.4
Hypoxic Control	23±2.9	3±0.7*	19±2.3	11±1.5
Early Treatment	25±2.6	3±0.3*	23+3.3	10±2.4*
Late Treatment	25±2.5	3±0.3*	26±2.6	16±2.4
Systemic Oxygen Uptake (mLO_2/kg/min)				
Sham	9±1.3	10±1.3	8±1.1	11±1.3
Hypoxic Control	7±1.0	2±0.6*	4±0.8*	7±0.5
Early Treatment	9±0.7	2±0.3*	4±0.8*	6±1.3*
Late Treatment	8±0.9	1±0.4*	3±0.4*	8±1.1

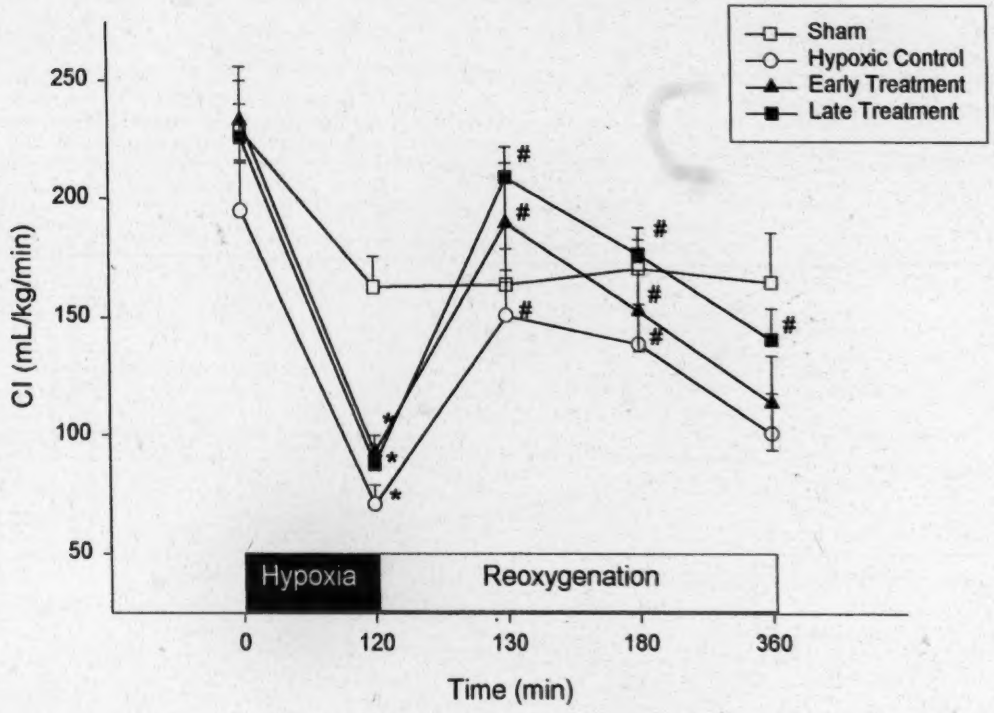
* $p<0.05$ vs. sham (one-way ANOVA)

Figure 7-1: Mean arterial pressure (MAP) during hypoxia-reoxygenation



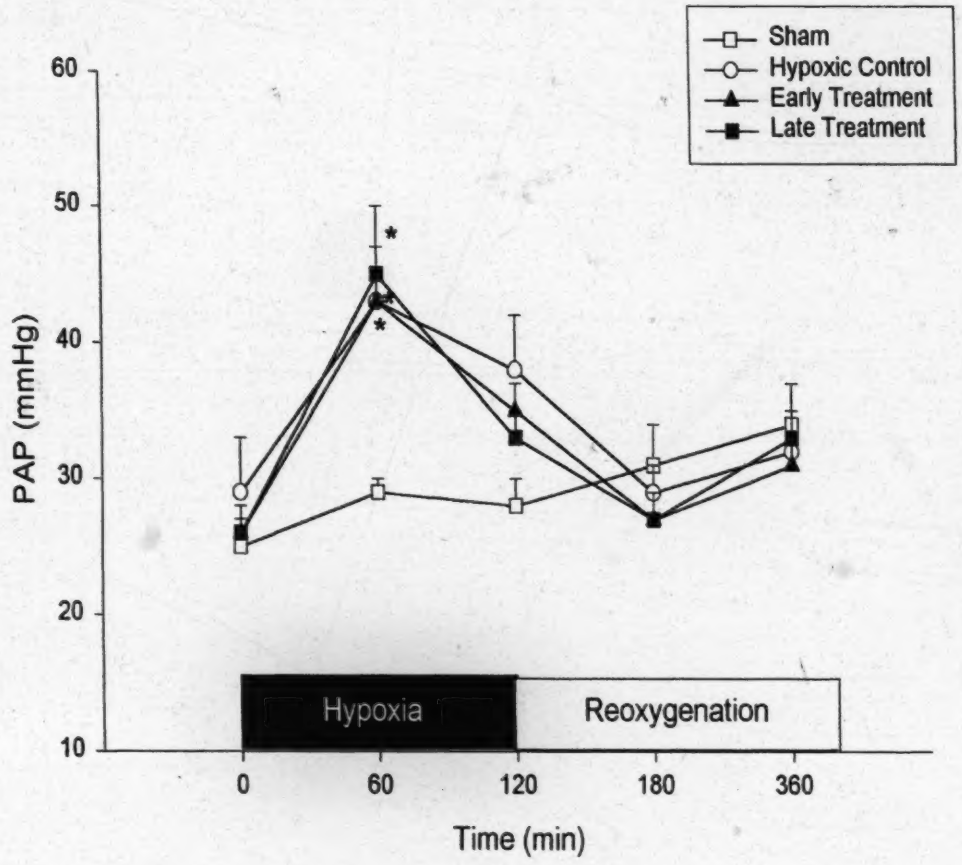
* $p<0.05$ vs. sham (one-way ANOVA)
# $p<0.05$ vs. respective end hypoxia value (one-way RM ANOVA)

Figure 7-2: Cardiac index (CI) during hypoxia-reoxygenation



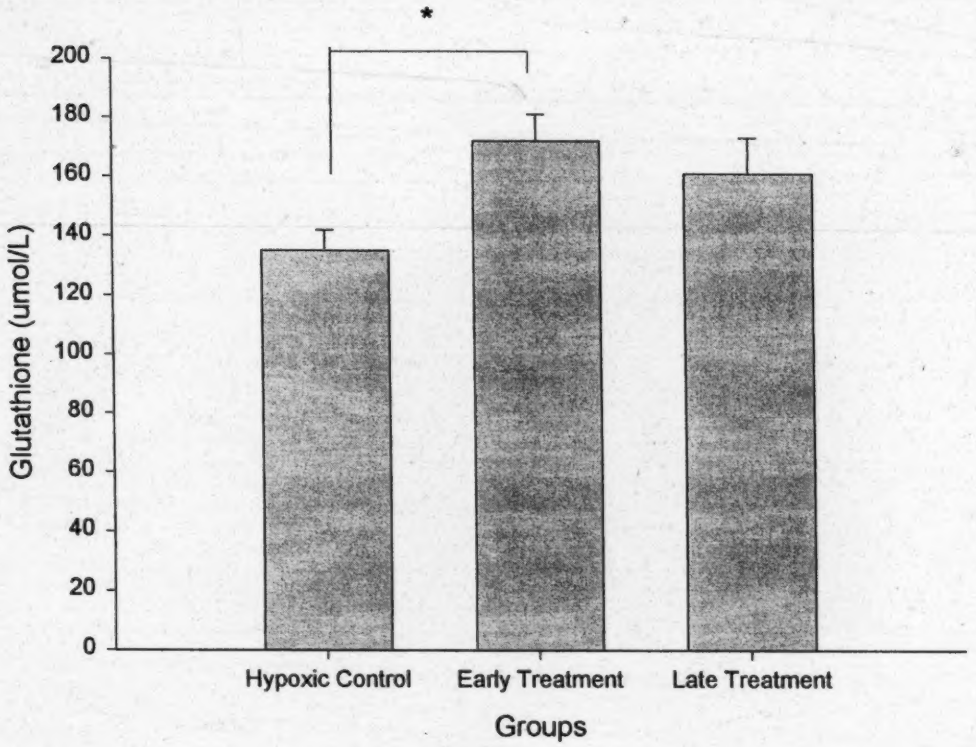
* p<0.05 vs. sham (one-way ANOVA)
# p<0.05 vs. respective end hypoxia value (one-way RM ANOVA)

Figure 7-3: Pulmonary artery pressure (PAP) during hypoxia-reoxygenation



* $p<0.05$ vs. sham (one-way ANOVA)

Figure 7-4: Left ventricle myocardial glutathione levels



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Chapter 8

N-Acetylcysteine Improves Renal Hemodynamics and Glutathione Stores in a Neonatal Piglet Model of Hypoxia-Reoxygenation

This chapter has been submitted for publication in the Am J Resp Crit Care Med

Chapter 9

Effects of Hypoxia-Reoxygenation and N-Acetylcysteine on the Gastrointestinal Tract

Introduction

Necrotizing enterocolitis can be a devastating gastrointestinal disease affecting the newborn infant. Over 2000 infants suffer from NEC annually in the United States. (1) While the precise underlying pathogenesis of NEC remains elusive, it is currently thought to be multi-factorial in origin. (2) Necrotizing enterocolitis has been associated with hypoxia and prematurity. (3) Pathologic lesions in NEC are consistent with an ischemic insult: infiltration of inflammatory cells with areas of mucosal edema, ulceration and necrosis. (4) However, despite pathologic evidence of ischemia, epidemiologic studies have yet to demonstrate a concrete link between hypoxemia and NEC. (2)

Following periods of tissue hypoxia, improvement of tissue perfusion subsequent to the restoration of tissue oxygenation may paradoxically exacerbate cellular injury. Such ischemia-reperfusion injury (IRI) has been documented in the bowel in different animal models. (5-7) Pathologic specimens from IRI experiments demonstrate lesions that resemble those seen in neonates with NEC. (6, 7) Similar bowel lesions have been demonstrated in animal models of asphyxia-reoxygenation. (8, 9)

Mucosal damage during the reoxygenation or reperfusion period has been linked to the increased generation of oxygen free radicals (OFR). (2, 8, 10) Oxygen free radicals are highly reactive species that contain a single unpaired electron. Consequently they are capable of interacting with and destroying various cellular components such as phospholipids, DNA, and mitochondria. (11) N-Acetylcysteine (NAC) is a thiol molecule and free radical scavenger that also serves as a precursor to

the main intracellular antioxidant, glutathione. (12) Previous animal studies have suggested NAC may reduce injuries associated with OFR. (13-15) Based on this information we examined the impact of NAC on the small intestine in a model of neonatal hypoxia reoxygenation injury. Our experiment was designed to test the hypothesis that the administration of NAC would attenuate intestinal hypoxia-reoxygenation injury.

Materials and Methods

Animals

Large white piglets (1-4 day old, 1.4-2.2 kg), were obtained from the university farm on the day of the experiment. All experiments adhered to the regulation of the Canadian Council of Animal Care and were approved by the Health Science Animal Welfare Committee at the University of Alberta. The minimum acceptable pre-operative arterial hemoglobin was 7 g/ dL.

Anesthesia and Preparation of Animals

Piglets were initially anesthetized with 5% isoflurane which was then titrated between 2-3% to ensure adequate anesthesia. A pulse oximeter (Nellcor, Hayward, CA) was used to monitor oxygen saturation. Temperature was monitored rectally and maintained between 38.5°C and 40°C with an overhead warmer and heating blankets. After an airway was surgically established inhalational anaesthesia was discontinued and mechanical ventilation initiated with pressure-controlled assisted ventilation (Sechrist infant ventilator model IV-100, Sechrist Industries Inc. Anaheim, CA) using

pressures of 20/4 cm H_2O and a rate of 18 breaths per minute. The fractionated inspired oxygen concentration (FiO_2) was set between 21-25% to maintain oxygen saturation between 88-95%. Oxygen concentration was measured by an Ohmeda 5100 oxygen monitor (Ohmeda Medical, Laurel, MD). Anesthesia was maintained with boluses of fentanyl (20 μg) and pancuronium (0.6 mg) followed by IV infusions of pancuronium (0.05-0.1mg/kg/hr), fentanyl (5-15μg/kg/hr), and midazolam (0.1-0.2mg/kg/hr). A single dose of acepromazine (0.25 mg) was given prior to the retroperitoneal dissection. Further boluses were given as necessary to maintain anaesthesia. Maintenance fluids throughout the experiment were 20 mL/hr of 10% dextrose and 4 mL/hr of normal saline. Blood samples in excess of what was required were returned to the pig. Total blood volume sampled through the experiment was approximately 8cc.

Surgical Procedure

The femoral artery and vein were exposed via a right groin incision. A double lumen catheter was placed in the femoral vein (3.5F or 5F Argyle™, Sherwood Medical Co, St Louis, MO) and advanced 15 cm to the level of the right atrium for infusion of intravenous (IV) fluid. A single lumen catheter was placed in the femoral artery, 3.5F or 5F Argyle™, and advanced 5 cm into the infra-renal aorta. Both lines were connected to pressure transducers for measurement of central venous pressure (CVP) and mean systemic arterial pressure (MAP) respectively. A tracheotomy was performed and a 3.5mm internal diameter endotracheal tube (Portex Inc, Wilmington, MA) was inserted into the trachea and mechanical ventilation was initiated. A left

anterior thoracotomy at the 4th interspace was performed. The pericardium was incised exposing the main pulmonary artery. A 6 mm Transonic® flow probe (6SB) was then placed around the pulmonary artery. The origin of the superior mesenteric artery (SMA) was exposed using a retroperitoneal approach. A 3 mm Transonic® flow probe (3SB) was placed around the SMA. The umbilical vein was identified, cannulated with a single lumen 5F catheter (Argyle™) and advanced into the portal vein. The catheter was then flushed with 1 mL of heparinized saline.

Hemodynamic Calculations

Cardiac output and SMA flows were corrected for individual pig weight and expressed in mL/kg/min as cardiac index (CI) and SMA flow index (SMAFI) respectively. Arterial oxygen content was estimated by the following: (1.36 X hemoglobin X oxygen saturation) + (0.002 X PaO_2). Mesenteric oxygen delivery was approximated by the product of SMAFI and arterial oxygen content. Percentage of cardiac output to the SMA was calculated by dividing SAMFI/CI * 100. Superior mesenteric artery flow as a percentage of baseline was obtained by dividing individual phase flow by the baseline flow and multiplying by 100.

Stabilization

After the surgical procedure all piglets were stabilized for 30-45 min. Inspired oxygen concentration (21-25%) and respiratory rate (12-22) were adjusted until the following criteria were met: PaO_2 60-100 mmHg, $PaCO_2$ 35-45 mmHg and pH >

7.35. Stability was defined as hemodynamic variation < 10% for 15 min while fulfilling the above blood gas criteria.

Monitoring

Oxygen saturation, heart rate (HR), MAP, CVP and SMA flows were continuously monitored and recorded throughout the experiment. Pressure and flow readings were digitized using a DT 2801-A converter board (Data Translation, ON) in a Dell 425E personal computer. Asyst custom software was used to continuously record all signals on a hard disk. Recordings at specific intervals were taken for two minutes and averaged for subsequent analysis.

Experimental Protocol

Piglets were block randomized into one of four groups: surgical sham, hypoxic control, early treatment with NAC and late treatment with NAC. The surgeon was unaware of the group assignment as treatment was provided by a laboratory assistant. At baseline a 1-2 cm portion of ileum was removed thru a small retroperitoneal window. The bowel ends were tied off with 3-0 silk and replaced into the peritoneal cavity.

The surgical sham was ventilated at 21% oxygen for 6hr. The remaining groups were subjected to 2hr of hypoxia by progressively decreasing the FiO_2 by increasing the amount of inhaled nitrogen. FiO_2 was manipulated to keep PaO_2 between 20 and 40 mmHg while reducing the cardiac output to 40% of baseline. After 2hr of hypoxia another ileal sample was taken. Piglets were then resuscitated

with 100% O_2 for 1hr followed by 21-25% O_2 for 3hr. Ileal tissue samples were taken at 10 min of reoxygenation. The IV NAC was prepared by diluting NAC (200 mg/mL) 1:1 in 10% dextrose. Treatment or placebo of equal volume 10% dextrose was then given. The early treatment group was given an IV bolus (150 mg/kg) of NAC at the time of reoxygenation. The late treatment group was given an IV bolus (150 mg/kg) of NAC 10 min into reoxygenation. Both NAC treatment groups received an IV infusion of NAC (100 mg/kg/hr) 30 min from the time of reoxygenation which was continued for the duration of the experiment. The infusion was prepared by diluting 5 mL of NAC (200 mg/mL) into 95 mL 10% dextrose. The hypoxic control received an equal volume placebo of 10% dextrose. At the conclusion of the experiment the piglet was euthanized with 240 mg IV pentobarbitol. Post-mortem examination confirmed placement of all catheters, flow probes and patency of vessels. Tissue samples from the ileum were removed, snap frozen and placed in liquid nitrogen. Additional samples were placed in 10% formalin for histological analysis.

Physiologic Measurements

All data was recorded in a blinded fashion. Baseline recordings of HR, MAP, CVP, pulmonary and SMA blood flows, arterial blood gas (ABL 500, Radiometer Medical, Denmark), arterial and mixed venous co-oximetry (OSM3 Hemoximeter, Radiometer Medical, Denmark) were taken. Hemodynamic, flow probe, arterial blood gas and venous co-oximetry measurements were recorded at 30 min intervals during hypoxia and at t=10, 30, 60, 120, 180, 240 mins of reoxygenation. In addition, small

bowel samples were taken at baseline, the end of hypoxia and at t=10 and 240 min of reoxygenation. During reoxygenation hypotension (MAP < 35 mmHg) was treated with a fluid bolus of 10 mL/kg which was repeated as necessary.

Biochemical Analysis

Small bowel levels of oxidized and reduced glutathione were determined using a glutathione assay kit (catalog number 703002, Cayman Chemical, Ann Arbor, MI). Briefly, frozen ileal tissue samples (50 mg ±10%) were homogenized in 500 µL of buffer containing 0.4 M 2-(N-morpholino) ethanesulphonic acid, 0.1M phosphate, and 2mM EDTA, at pH 6.0 (MES). The sample was then centrifuged at 10,000 g for 15 min at 4°C. The supernatant was removed and stored at 4°C. Deproteination of all samples was accomplished using a combination of 10% metaphosphoric acid and 4M triethanolamine. A microplate assay was then done by adding glutathione reductase, $NADP^+$, MES buffer, water and 5,5'-dithiobis-2-nitrobenzoic acid. Samples were incubated in the dark on an orbital shaker and absorbance was measured at 25 min at 405 nm using a microplate reader (Spectra Max 190, Molecular Devices, Sunny Vale, CA). The total glutathione concentration was calculated using a standard curve. Oxidized glutathione was measured by mixing deproteinated samples with 1 M 2-vinylpyridine and incubating for one hour at room temperature. Samples then underwent the same microplate assay as described above. Levels of reduced glutathione were determined by subtracting oxidized glutathione (GSSG) from the total glutathione levels. Tissue glutathione and GSSG contents were expressed as

umol/g protein. Tissue protein concentration was assayed by bicinchoninic acid method using albumin as standards.

Histology

Tissue samples were collected at the defined phases of the experiment and placed in 10% formalin. Tissue was embedded in paraffin blocks, sectioned and stained with hematoxylin and eosin. Two pathologists, (Graham Slack, Lawrence Jewell) who were blinded to the randomization, independently analyzed slides. Histologic injury was assessed using Park's criteria as follows: 0-normal, 1-subepithelial space at villus tip, 2-extended subepithelial space, 3-epithelial lifting along villus sides, 4-denuded villi, 5-loss of villus tissue, 6-crypt layer infarction, 7-transmucosal infarction, 8-transmural infarction. (7)

Data Analysis

Data was analyzed using SigmaStat2.0 for windows (Jandel Corporation, San Rafael, CA). All results are expressed as mean ± standard error of mean. Experimental groups were compared using one-way analysis of variance (ANOVA). Post hoc analysis was completed using the Fisher LSD test. Correlation was analyzed using the Pearson Product Moment test. Histologic scores were compared using the Fisher Exact test. For all analyses significance was defined as $p < 0.05$.

Results

Twenty-four piglets were instrumented and randomized into 4 groups. There were no differences in baseline hemodynamic characteristics between groups (Table 9-1). After 2hr hypoxia (FiO_2 0.11±0.01, PaO_2 32±2 mmHg) all pigs were acidotic

(mean pH ranging between 6.95 and 6.98) hypotensive and in cardiogenic shock. During reoxygenation hemodynamics recovered and acidosis corrected. There were no significant differences in the total volume or number of fluid boluses between groups (25±8 vs. 20±5 vs. 8.3±3 mL/kg for control, early and late treatment respectively).

There was also no difference in baseline SMAFI between groups. All piglets subject to hypoxia underwent a progressive reduction in SMAFI. After 60 min of hypoxia SMAFI ranged between 63-69% of baseline (vs. 75% of baseline for the sham, $p>0.05$) (Figure 9-1). Progression of hypoxia resulted in further reduction of SMAFI while the surgical sham remained stable at 75% of baseline. After 2hr of hypoxia, the SMAFI were significantly reduced and between 29-36% of baseline (vs. 75% in the surgical sham, $p<0.001$). Absolute flows in the hypoxic control, early and late treatment groups were significantly reduced compared to the surgical sham (17±3, 17±3 and 23±4 vs. 33±3 mL/kg/min, respectively, $p<0.05$). Flow improved immediately following reoxygenation and ranged between 55-89% of baseline at 10 min reoxygenation. After this SMAFI declined slowly for the duration of the experiment in all 3 hypoxic groups while remaining stable in the surgical sham. After 4hr reoxygenation, SMAFI of both the control and early treatment group were significantly different from the surgical sham (36±11 and 26±8 vs.91±24 % of baseline, respectively, $p<0.05$) and the late treatment group (51±14 % of baseline) was not significantly different from the sham.

A large proportion of the CI was dedicated to the SMA during all phases of the experiment (Figure 9-2). Changes in CI were reflected in SMAFI throughout the experiment. At baseline between 22-26% of the cardiac index was directed to the SMA. After 30 min hypoxia 19-22% was dedicated to the SMA, after 2hr hypoxia 19-27% went to the SMA, similar proportions were observed at 10 min reoxygenation while after 4hr reoxygenation between 17-23% of the cardiac index was directed to the SMA. There were no differences in the SMAFI percent of CI between groups at any point during the experiment.

Oxygen delivery to the bowel was similar among all groups at the start of the experiment (Table 9-2). After 2hr hypoxia there was a significant reduction in mesenteric oxygen delivery (11-13% of baseline) compared to the surgical sham (0.6±0.1, 0.6±0.1, 0.8±0.1 vs. 3.8±0.4 mLO_2/kg/min for hypoxic control, early and late treatment groups vs. sham respectively, $p<0.05$). Reoxygenation resulted in an immediate increase in oxygen delivery, which then declined over the course of the experiment. Among the treatment groups, after 4hr reoxygenation there was a trend to increased oxygen delivery in the late treatment group, $p=0.065$, $\beta=0.61$, (Table 9-2).

Baseline levels of GSH and GSSG were not different between groups (7.9±2, 5.5±2, 5.8±2 umol/g protein for control, early and late treatment respectively, $p>0.05$). Two hours of hypoxia resulted in a reduction in intestinal tissue GSH levels while levels of GSSG remained the same or decreased slightly. Although not significant, after 4hr reoxygenation, there was an increase in tissue GSH levels in both treatment groups over baseline levels ($p>0.05$), while GSH levels in the control were not different from baseline (Figure 9-3). There was no difference in the

intestinal redox ratio (GSH:GSSG) between groups at any point in the experiment (data not shown).

Histological changes in the small bowel were observed in this experiment. Using the Park's grading scale intestinal sections ranged from normal to showing evidence of denuded villi (Figure 9-4). Although there was no significant difference in Park's staging between experimental groups the late treatment group had more normal histologic samples (4) than the early treatment (1) or control (2) ($p > 0.05$). There was no correlation between intestinal GSH levels and level of histologic injury, SMAFI or mesenteric oxygen delivery.

Discussion

As shown in other models, severe hypoxia created a reduction of SMAFI and oxygen delivery. (8, 16, 17) However, in contrast to some other experiments SMAFI did not reach supra-baseline levels upon reoxygenation. SMA flows approximated baseline flows after 10min reoxygenation then slowly declined for the remainder of the experiment. This may have been related to the severity of the hypoxic insult. One study by Nowicki et al (16) demonstrated similar findings which the authors speculated were secondary to vascular damage during reoxygenation. Other experiments have used varying degrees of hypoxia to generate systemic pH in the range of 7.06-7.36. (8, 16, 17) In our hypoxic cohort the mean pH after two hours of hypoxia was between 6.95 and 6.98. After an initial recovery, decreasing SMAFI in the hours following reoxygenation may have clinical implications. Reduced SMA

flows increase the risk for further IRI of the bowel and could increase the risk of activating a cascade for the development of NEC.

Human neonates born prematurely have an incompletely developed response to the detoxification of OFR. (18, 19) Similarly, newborn piglets have a decreased response to OFR. Levels of catalase and glutathione peroxidase, both enzymes functioning in an antioxidant capacity, have been shown to be low in intestinal tissue of newborn piglets. Levels of GSH, an intracellular antioxidant, were significantly lower in 1-day-old piglets when compared to 1-month-old piglets. (20) While some have demonstrated a reduction in antioxidant defence, other investigators have documented high concentrations of free radical producing enzymes. Studies on pig and rat intestinal tissue have shown high levels of the OFR generating enzyme xanthine oxidase. (21, 22) The combination of underdeveloped antioxidant defence and high levels of oxygen radical generating enzymes places the newborn at increased risk for oxidant-induced damage.

N-Acetylcysteine was used in this experiment in an attempt to increase the antioxidant capacity of the neonatal intestine to reduce the morbidity associated with ischemia reperfusion injury. In our experiment two hours of hypoxia resulted in a reduction in GSH levels compared to the surgical sham and after 4hr reoxygenation both the early and late treatment groups had GSH levels above baseline, although the differences did not reach statistical significance. This suggests that NAC did have some impact on cellular GSH content. The late treatment group also had more intestinal samples with no damage (4) in comparison to the control (2) and early treatment groups (1), and fewer samples with higher grades of injury suggestive of a

protective effect. Delivery of NAC to the intestinal villi may have been sub-optimal by reduced SMA blood flows. N-Acetylcysteine delivery to enterocytes may be improved by intra-luminal administration as opposed to intravenous as in our experiment.

Other experiments have had mixed results with interventions against OFR mediated bowel injury. Granger et al (23) used inhibitors of xanthine oxidase to reduce ischemia induced intestinal permeability in a feline model. One study of ischemia-reperfusion in rats treated with NAC documented a reduction in intestinal injury as measured by histology and immunohistochemical staining. (14) Another ischemia-reperfusion experiment in newborn pigs did not show any benefit with pretreatment using the OFR scavengers superoxide dismutase and catalase. (6)

Necrotizing enterocolitis remains a difficult clinical problem. The underlying pathologic lesion is consistent with an ischemic insult, however, it is uncertain if intestinal ischemia is the primary underlying pathology. (4) Rather, epidemiologic and experimental data support the theory that NEC is multi-factorial in etiology. (2, 24)

Experimental models using piglets exposed to hypoxia have previously documented histologic intestinal changes consistent with NEC. (8, 9) Histologic changes were evident in the bowel samples from our experiment with many sections showing evidence of ischemic damage. Removing multiple ileal samples over the course of 4hr may have confounded some of the histology; ligation of segmental blood supply could have created venous congestion.

Our model does have some limitations. Necrotizing enterocolitis is associated with prematurity while our experiments were done on term 1-3 day old piglets. While our hypoxia-reoxygenation protocol did induce some intestinal ischemic changes it was not uniform. Several piglets exposed to 2 hr hypoxia did not show any histologic evidence of ischemia. A protocol generating reliable ischemic intestinal changes in all pigs subject to hypoxia could improve the assessment of intervention strategies.

Treatment with NAC did have an impact on the gastrointestinal tract. Late treatment brought the SMAFI close to the surgical sham and had more histologic sections without evidence of ischemic damage. After 4hr reoxygenation, treatment with NAC also increased cellular levels of GSH to values above baseline. N-Acetylcysteine shows promise for attenuating the damage associated with hypoxia and reoxygenation but requires more study to further define its role.

Table 9-1: Baseline hemodynamic and physiologic characteristics*

	Surgical Sham	Hypoxic Control	Early Treatment	Late Treatment
HR (bpm)	203±16	220±11	239±7	217±12
MAP (mmHg)	70±5	63±5	68±1	71±4
CI (mL/kg/min)	229±27	195±20	233±17	226±14
SMAFI (mL/kg/min)	51±10	54±8	58±5	69±9
pH	7.39±0.02	7.39±0.01	7.39±0.01	7.40±0.01
$PaCO_2$ (mmHg)	39±1	40±1	41±1	40±2
PaO_2 (mmHg)	68±2	79±10	67±2	64±2

*No differences between groups

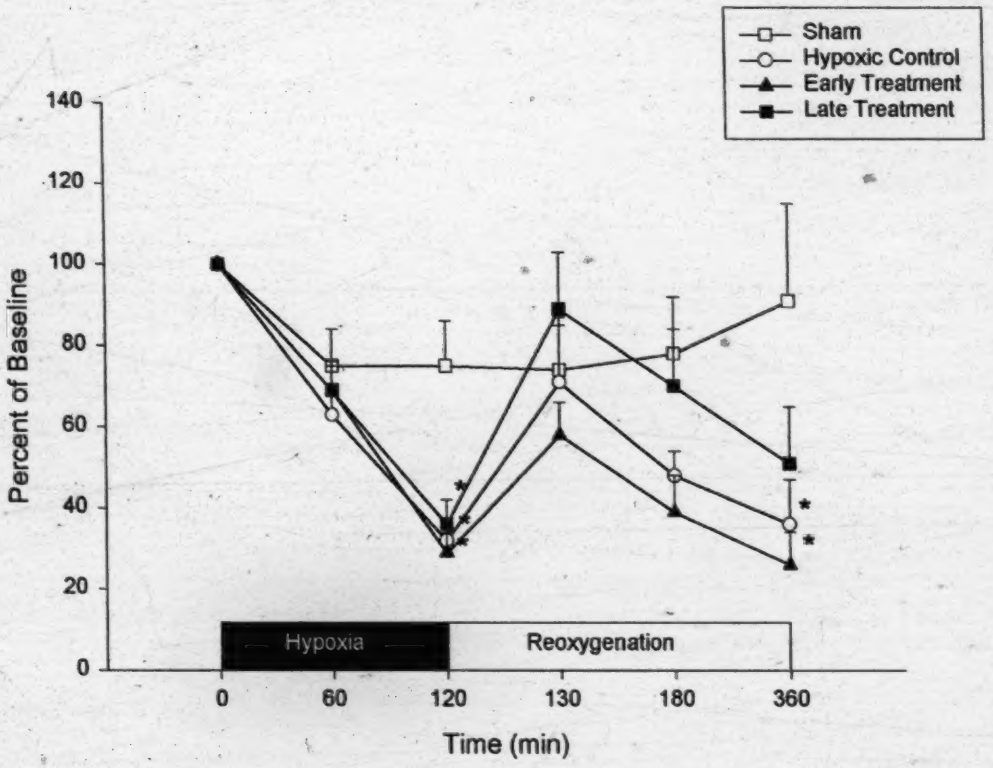
HR- heart rate, bpm- beats per minute, MAP- mean arterial pressure, CI- cardiac index, SMAFI- superior mesenteric artery flow index, SMA DO_2- superior mesenteric artery oxygen delivery

Table 9-2: Mesenteric oxygen delivery ($mLO_2/kg/min$) during hypoxia-reoxygenation

	Sham	Hypoxic Control	Early Treatment	Late Treatment
Baseline	5.8±1.2	6.2±1.0	6.3±0.6	8.1±1.5
End hypoxia	3.8±0.4	0.6±0.1*	0.6±0.1*	0.8±0.1*
10 min reoxygenation	3.7±0.2	5.4±1.1	4.9±0.9	8.1±1.3*
4 hr reoxygenation	4.2±0.6	2.0±0.7	1.6±0.6	4.3±1.3

* $p<0.05$ vs. sham (one-way ANOVA)

Figure 9-1: SMA flow as percent baseline during hypoxia-reoxygenation



* $p<0.05$ vs. sham (one-way ANOVA)

Figure 9-2: SMA flow as percent cardiac index during hypoxia-reoxygenation

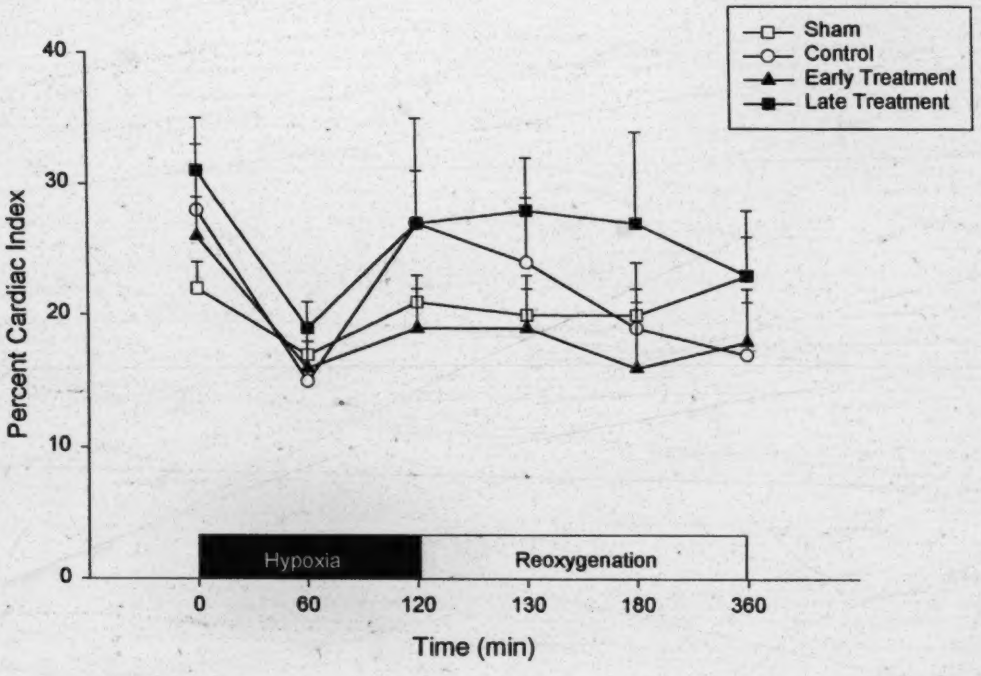


Figure 9-3: Bowel Glutathione content as percent of baseline values during hypoxia-reoxygenation

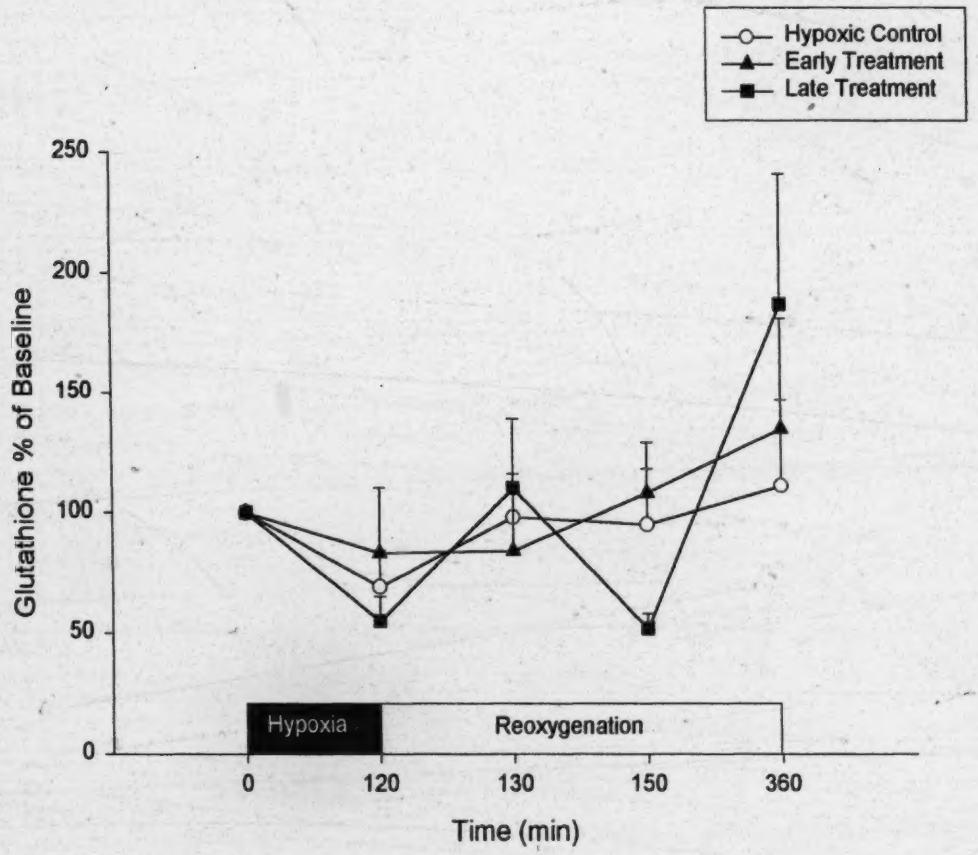
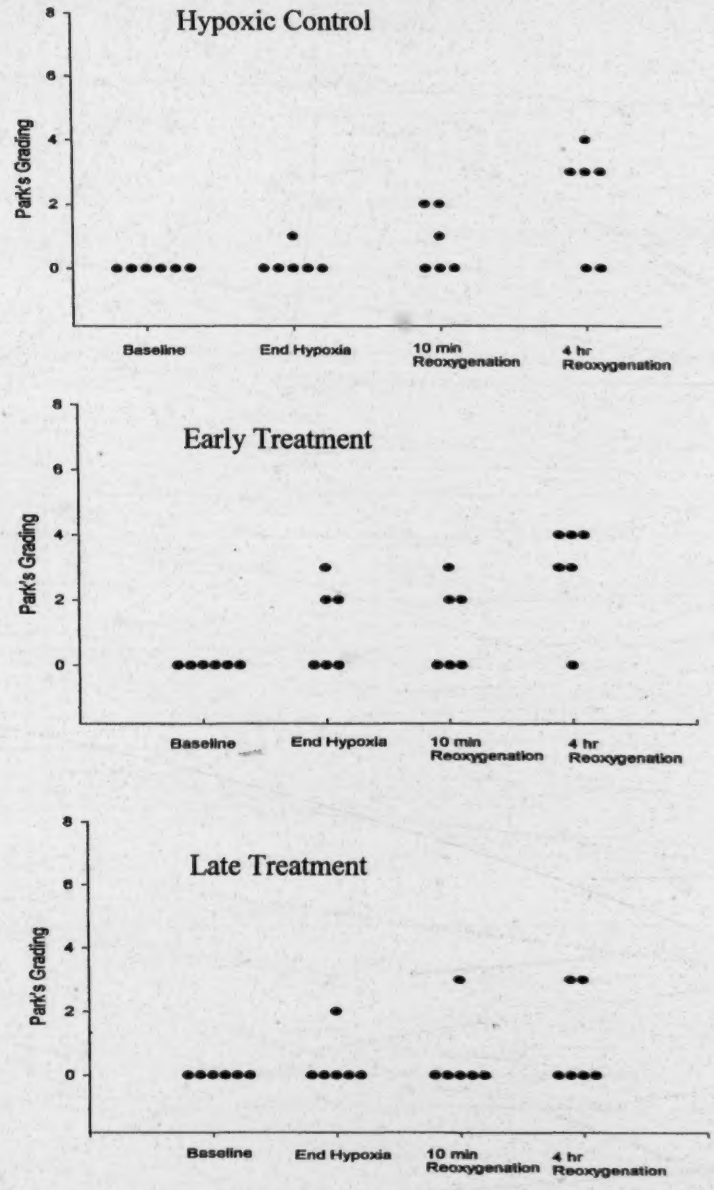


Figure 9-4: Park's histologic grading of intestinal injury



Park's Criteria for histologic assessment of intestinal injury: 0 = normal mucosa, 1 = subepithelial space at villus tip, 2 = extended subepithelial space, 3 = epithelial lifting along villus sides, 4 = denuded villi, 5= loss of villus tissue, 6= crypt layer infarction, 7=transmucosal infarction, 8=transmural infarction.

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Chapter 10

Carotid Hemodynamics in Hypoxia-Reoxygenation and Treatment With N-Acetylcysteine

Introduction

Neonatal asphyxia is a common clinical condition. Birth asphyxia has been defined as: umbilical arterial pH<7, Apgar score < 3 at 5 minutes, neurologic manifestations and multi-system organ involvement. (1) The etiology of birth asphyxia ranges from maternal hypotension to utero-placental insufficiency to congenital malformations of the cardio-respiratory system. Neonatal asphyxia is associated with a range of neurologic disabilities including hypoxic-ischemic encephalopathy, cerebral palsy, mental retardation and epilepsy. (2) Hypoxic-ischemic encephalopathy is a spectrum of clinical findings that can range from a short duration of hyper alertness and exaggerated reflexes to brainstem dysfunction, coma and death. (3)

Cerebral blood flow responds to alterations in several physiologic variables. Cerebral metabolic rate, arterial oxygen content and carbon dioxide levels can all affect cerebral perfusion. (4) In a normoxic state cerebral blood flow is maintained during episodes of hypotension via dilation of intra-cerebral arteries and arterioles. Similarly, the brain adapts to periods of hypoxia by increasing oxygen extraction and vasodilating arteries to increase blood flow. (4) Severely asphyxiated neonates, often hypotensive and hypoxic, have difficulty delivering adequate amounts of oxygen to the brain despite vasodilation. Reduced oxygen delivery places these neonates at risk for cerebral damage.

Some work has implicated glutamate, an excitatory neurotransmitter, in cases of hypoxia induced neuronal damage. (2) Recently more attention has been focused on the role of oxygen free radicals (OFR) in the genesis of post-asphyxia neurologic

disorders. (5, 6) Oxygen radicals (species containing a single unpaired electron) have been linked to both neuronal necrosis and apoptosis. (7) With a high concentration of polyunsaturated phospholipids, the brain is also susceptible to lipid peroxidation by OFR. Success using free radical degradative enzymes (superoxide dismutase and catalase) in attempt to reduce OFR mediated brain injury has been limited. (2, 8)

N-Acetylcysteine (NAC) is a well-known antioxidant. It is able to directly neutralize free radicals via its sulfhydryl group or alternatively enhance cellular antioxidant capacity by increasing intracellular glutathione stores. (9) N-Acetylcysteine has been used in clinical medicine for over 25 years. (10) We designed an experiment to test the impact of NAC on carotid artery blood flow in a piglet model of neonatal hypoxia-reoxygenation.

Materials and Methods

Piglets were anesthetized, instrumented and subjected to the experimental protocol as previously described on page 86. There were four experimental groups; surgical sham, hypoxic control, early treatment with NAC and late treatment with NAC. Notably the right common carotid artery was exposed and isolated. A 2 mm flow probe (Transonic® Systems Inc., Ithica, NY, model 2SS) was then placed around the artery. Carotid flows were recorded at baseline, at 30 minute intervals during hypoxia, and at 0, 10, 60 and 240 minutes of re-oxygenation. Pulmonary artery flows were recorded with a 6 mm flow probe (Transonic®, model 6SB) placed around the pulmonary artery. Flow readings were digitized using a DT 2801-A converter board (Data Translation, ON, Canada) in a Dell 425E personal computer.

Asyst custom software was used to continuously record all signals on a hard disk. Recordings at specific intervals were taken for two minutes and averaged for subsequent analysis.

Hemodynamic Calculations

All blood flows were indexed by dividing by body mass in kilograms. Carotid vascular resistance index was estimated by: {mean arterial pressure (MAP) – central venous pressure (CVP)} / carotid index. Carotid flows as a percentage of cardiac output were calculated by: (carotid index / cardiac index) X 100%. Carotid flow as a percentage of baseline was obtained by: (specific phase flows / baseline flow) X 100%. Carotid oxygen delivery was estimated by multiplying carotid flow index by arterial oxygen content. Arterial oxygen content was calculated in the following manner: (1.36 X hemoglobin X oxygen saturation) + (0.002 X PaO_2).

Data Analysis

Data was analyzed using SigmaStat2.0 for windows (Jandel Corporation, San Rafael, CA). Differences between means of experimental groups were compared using one-way ANOVA with post hoc analyses by Tukey test. Significance was defined as $p < 0.05$. All results are expressed as mean ± standard error of mean unless otherwise stated.

Results

Baseline carotid flows were not different between groups. After 30 min of hypoxia carotid flows increased in both the hypoxic control and the late treatment cohort, while the early treatment group remained at baseline (117±7, 117±3 and

100±15% of baseline respectively) (Figure 10-1 and 10-2). At the same time point the surgical sham had carotid flows 95% of baseline. After 2h of hypoxia there was a trend to reduced absolute carotid flows in the groups subjected to hypoxia when compared to the surgical sham, $p= 0.055$, $\beta= 0.6$ (Figure 10-1). After 2 hr of hypoxia carotid flows fell below baseline (86±6, 58±13, 45±6, 58±5% of baseline for sham, hypoxic control, early and late treatment respectively). Reoxygenation immediately improved carotid flows. After 10 min of re-oxygenation there were no differences between the 4 groups. Over 4 hr flows then gradually declined without any differences between groups at the end of the experiments.

At baseline the proportion of cardiac output destined for a single carotid artery ranged between 13-17% for the four groups without any differences between groups (Figure 10-3). After 30 min of hypoxia the treatment groups ranged between 12-17% while the surgical sham remained steady at 13%. Two hours of hypoxia increased the range of cardiac output destined for the right carotid to between 14-26%. Again there were no differences between groups. Ten minutes into reoxygenation the range tightened between 10-16%, while at 4 hr the range was 11-19%. There were no differences between treatment groups at either 10 min or 4 hr of reoxygenation.

Calculated carotid resistance index did not differ between groups at baseline. There were no differences between treatment groups at any point during hypoxia or reoxygenation (Table 10-1).

Calculated carotid oxygen delivery declined during hypoxia. After 2 hr the surgical sham has a significantly higher oxygen delivery than all groups subject to hypoxia (2.8±0.2 vs. 0.62±0.1, 0.47±0.1, 0.67±0.1 mLO_2/kg/min, for sham, control,

early and late treatment respectively, $p<0.05$) (Table 10-1). After 10 min resuscitation with 100% oxygen there was a trend to higher oxygen delivery in the groups subject to hypoxia-reoxygenation compared to the surgical sham, ($p=0.05$), with no differences between treatment groups. After 4hr with the piglets in 21% oxygen there was no difference in oxygen delivery between treatment groups or between treatment groups and the surgical sham.

Discussion

Neonatal asphyxia is a complex multi-system disorder. Severely asphyxiated newborns can suffer from neurologic morbidities for the remainder of their life. (3) Unfortunately, it is often difficult to determine, in a timely fashion, which newborns have been severely asphyxiated. Different diagnostic and biochemical modalities have been used in an attempt to predict outcomes. (11-13) Nagdyman et al found serum levels of CK-BB and protein S-100 to be elevated 2 hr after birth in severely asphyxiated infants when compared to age matched controls. When combined with blood gas pH < 6.9 and base deficit > 17 the predictive value of these markers increased. (14) These findings are significant as identifying neonates at risk for asphyxiated related brain injury is the first step in developing effective treatments.

Interventions against OFR mediated neurologic injury have been attempted in the past. (2) In one clinical study asphyxiated infants were randomized to either treatment with allopurinol or a control group. The group treated with allopurinol had a significantly reduced level of prooxidant stress as measured by serum non-protein

bound iron. The treatment group also had an attenuated increase in malondialdehyde, a product of lipid peroxidation. (15)

One study investigating the effects of severe asphyxia on cerebral blood flow, measured by xenon 133 clearance, documented a loss of autoregulation in newborns with cerebral damage. (16) Impairment of cerebral autoregulation puts the newborn brain at risk of further ischemic injury.

Our model looked primarily at carotid blood flows and the effects of NAC on carotid blood flows. Not surprisingly, 30 min of hypoxia resulted in a maintenance or increase in carotid blood flows as a compensatory mechanism for reduced arterial oxygen content. The normal cerebrovascular response to hypoxia is vasodilation coupled with increased oxygen extraction. (4) If we had measured segmental cerebral blood flow one can speculate that certain areas of the brain would have undergone a dramatic increase in blood flow. In a study of fetal lambs exposed to hypoxia there were differences in regional cerebral blood flow, with higher proportional increases in the brainstem when compared to the cortex and subcortex. (17) Using radiolabelled microspheres, Rootwelt et al (18) documented increased blood flows in the forebrain and brainstem but not in the cerebellum of newborn pigs during hypoxia. During reoxygenation blood flow was significantly increased to all regions of the brain.

One limitation of our experiment is the assumption that carotid arterial flows correlate to cerebral blood flow. Studies in fetal lambs have shown that carotid arterial flows and cerebral blood flow are closely related even at extremes of oxygenation. (19) Another study in fetal sheep documented a modest correlation between carotid flows and cerebral blood flow. However, there was a strong

significant correlation between changes in carotid flows and changes in cerebral flows, suggesting that any alterations in carotid flows mirror changes in cerebral perfusion. (20)

In our model, when carotid flows were corrected for cardiac output there were no differences between groups at baseline or any point during hypoxia. Similarly, during the reoxygenation period there were no differences between groups. During hypoxia we had predicted that there would be a significant increase in the proportion of cardiac output to the carotid artery as a compensatory mechanism for decreased arterial oxygen content. This did occur in both the hypoxic control and late treatment group without reaching statistical significance. As our sample size was calculated to evaluate systemic effects like cardiac output, it is possible that our study lacked the power to detect regional differences.

N-Acetylcysteine has been proven safe and efficacious in a variety of clinical situations. (10, 21) In our experiments NAC did not have any regional hemodynamic effects. There were no differences in carotid flows between groups at any point during the experiment. Between groups there were also no differences in the proportion of cardiac output dedicated to the carotid artery. Along similar lines there were no differences in carotid artery oxygen delivery between treatment groups.

Our results provide further data on the safety profile of NAC in the setting of regional circulation. Further study is required to investigate if NAC is able to reduce oxidant stress on the brain and decrease the resultant brain injury.

Table 10-1: Common carotid vascular resistance and oxygen delivery during hypoxia-reoxygenation

	Baseline	End Hypoxia	10 min Reoxygenation	4 hr Reoxygenation
Carotid Resistance Index [mmHg/ (mL/kg/min)]				
Sham	2.4±0.2	2.4±0.3	2.4±0.3	2.2±0.3
Control	1.9±0.2	1.6±0.3	2.0±0.2	3.2±0.8
Early Treatment	2.3±0.3	1.7±0.3	2.5±0.4	2.1±0.3
Late Treatment	2.1±0.1	1.1±0.1	2.0±0.2	2.5±0.4
Carotid Oxygen Delivery (mLO_2/kg/min)				
Sham	3.3±0.3	2.8±0.2	2.8±0.2	2.1±0.3
Control	3.7±0.2	0.6±0.1*	3.6±0.1	1.3±0.3
Early Treatment	3.3±0.4	0.5±0.05*	3.0±0.4	1.4±0.2
Late Treatment	3.7±0.3	0.7±0.08*	3.8±0.3	1.9±0.5

* $p<0.05$ vs. sham (one-way ANOVA)

Figure 10-1: Common carotid flow plotted over the course of hypoxia-reoxygenation

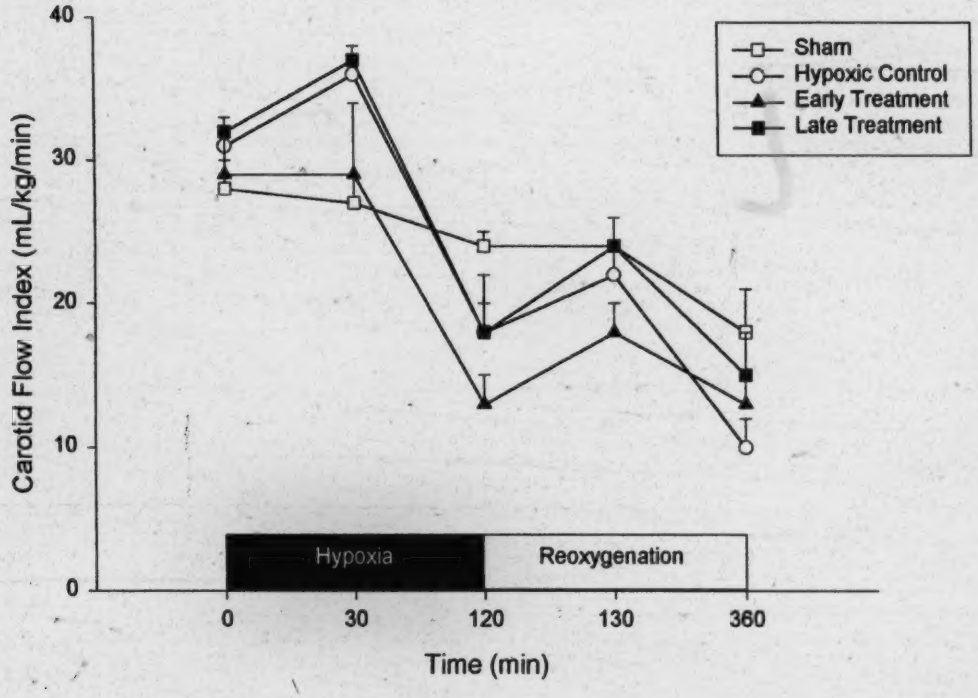


Figure 10-2: Common carotid flows as a percentage of baseline values during hypoxia-reoxygenation

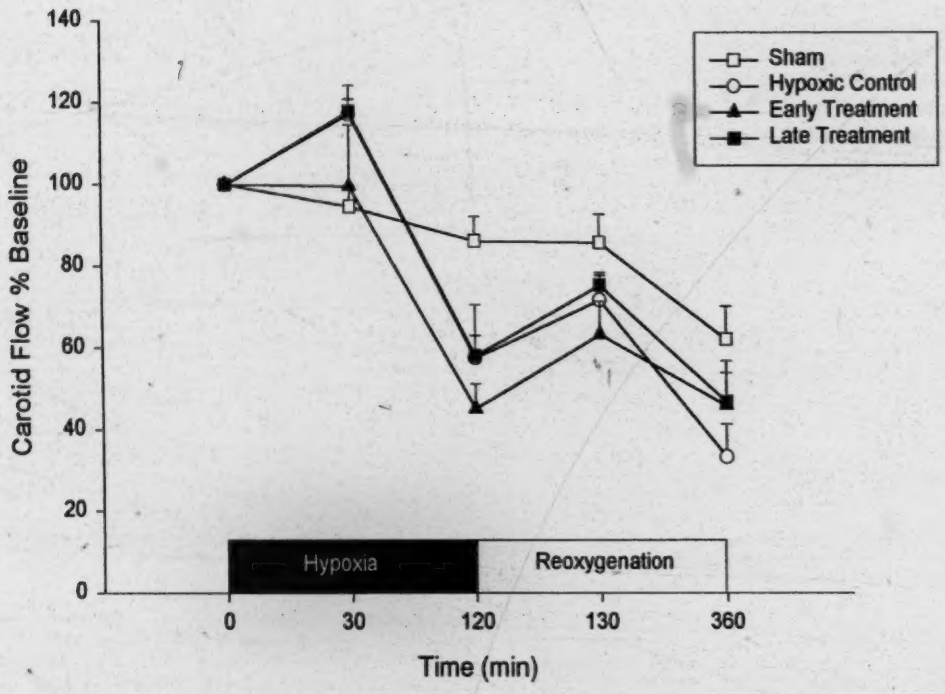
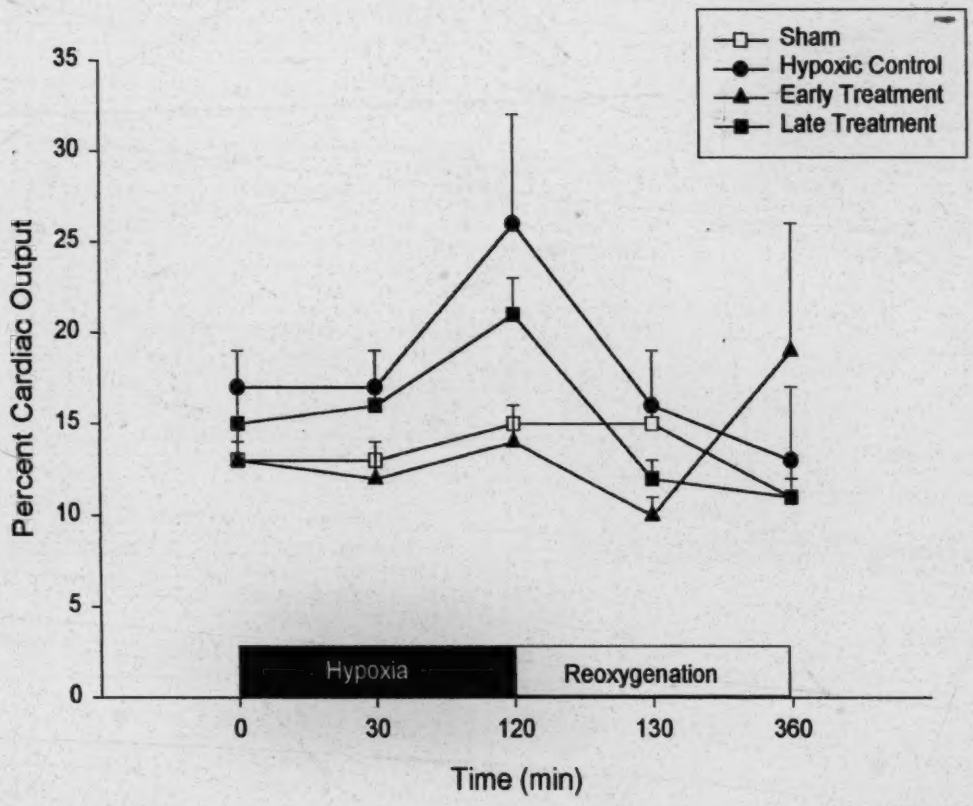


Figure 10-3: Common carotid flows calculated as a percentage of cardiac output during hypoxia-reoxygenation



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Chapter 11

Conclusions

Neonatal asphyxia remains a vexing clinical problem. Advances in neonatal critical care have facilitated the resuscitation of newborns. Despite these advances, many neonates will suffer from asphyxia and its associated morbidities. Paradoxically, the resuscitation itself may create additional morbidities secondary to the generation of OFR. Previous investigators have documented large increases in OFR generation that correlated with organ dysfunction. Intervention attempts to reduce the generation of OFR have been met with mixed results. Consequently, we set out to investigate the effects of using an antioxidant, NAC, in an attempt to reduce the generation of free radicals in our model of neonatal hypoxia-reoxygenation. We hypothesized that by reducing OFR generation we would be able to reduce end organ injury.

During the course of reoxygenation and at the conclusion of the experiment there were no detrimental hemodynamic effects in either systemic or regional circulation in the NAC treatment groups. Our experiments add to the body of evidence supporting the safety of NAC in a novel clinical application: neonatal asphyxia. With new knowledge that NAC does not negatively affect hemodynamics we can investigate its effects in different neonatal conditions.

Not surprisingly, systemic hemodynamics were severely compromised during hypoxia. Reoxygenation restored hemodynamics to near baseline levels. Treatment with NAC 10 min into reoxygenation improved systemic hemodynamics and oxygen delivery as measured by MAP, CI and DO_2. Similar effects were not observed in the group given NAC at the time of reoxygenation. Unfortunately there was no evidence of histologic ventricular damage in any group so we were unable to extrapolate

improved systemic hemodynamics to reduced end organ damage. N-Acetylcysteine did result in increased levels of glutathione in the left ventricle when compared to controls. Both treatment groups increased cellular glutathione, however, only the early treatment group was significantly higher than the hypoxic control. These results are exciting in that they demonstrate a treatment effect; NAC improves cardiac levels of glutathione. Further experiments will be necessary to fully delineate the clinical implication.

Regional hemodynamics were also depressed during hypoxia and recovered during reoxygenation. Carotid flows were not different between treatment groups. After 4 hours reoxygenation renal blood flows were significantly higher in the late treatment group compared to the hypoxic control. Renal tissue levels of glutathione were also significantly increased in the late treatment group. These results suggest that our treatment was effective at preserving renal blood flow and increasing the antioxidant reserves of the kidney. This is a significant finding as preservation of renal blood flow may result in reduced rates of renal failure, a common complication in asphyxiated newborns.

Mesenteric hemodynamics followed a similar pattern of significant depression during hypoxia followed by a recovery during reoxygenation. Once again late treatment with NAC resulted in improved SMA flows and a trend to increased oxygen delivery. There were also fewer piglets with histologic bowel injury in the late treatment group.

Unfortunately the experiment was powered to detect changes in cardiac output. Consequently, the experiment was underpowered to detect significant differences in regional circulation, glutathione levels and histology.

N-Acetylcysteine has proven effective in reducing some of the hemodynamic and biochemical effects of hypoxia-reoxygenation. The findings in our study necessitate further investigation in a clinical setting. Treatment with NAC 10 minutes into reoxygenation seemed to provide the greatest protective effect. This is an encouraging result as intervening at the exact moment of reoxygenation is not clinically practical. However, giving a treatment after the neonate has been stabilized is reasonable in a clinical environment. In the absence of any evidence of detrimental hemodynamic effects the role of NAC in neonatal asphyxia will best be delineated in a clinical trial.